



# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 95

NUMBER 1

September 1975

Contents

- 1 Eriksson, H.-W., Melström and A. Ryrfeldt  
The Biliary Excretion of  $^3\text{H}$  Insulin and  $^3\text{H}$  Terbutaline in the Unanesthetized Rat
- 6 Steen, J. B. and S. Stray-Pedersen  
The Permeability of Fish Gills with Comments on the Diurnal Behaviour of Cellular Membranes
- 21 Wellenberg, L. R.  
Effect of Cold Exposure on Free Water Reabsorption in Hydropic Man
- 28 Gustafsson, L., P. Hedqvist and H. Lagerstedt  
Potentiation by Prostaglandins  $\text{E}_1$ ,  $\text{E}_2$  and  $\text{F}_{2\alpha}$  of the Contraction Response to Transmural Stimulation in the Bovine Irs Sphincter Muscle
- 34 Auerbach, P., J. Kertzen and G. Be  
Effects of Catecholamine-Infusions and Hypoxia on Pulmonary Blood Volume and Extra-vascular Lung Water Content in Cats
- 41 Blix, A. S.  
The Importance of Aspiration for the Development of Diving Bradycardia in Ducks
- 46 Björkme, L., L. Kalljeer, A. Marthé and G. Birke  
Specific and Unspecific Removal of Circulating Noradrenaline in Pulmonary and Systemic Vascular Beds in Man
- 64 Kinnula, V. L.  
Rat Liver Mitochondrial Enzyme Activities in Hypoxia
- 69 Alm, A.  
Radioactively Labelled Microspheres in Regional Cerebral Blood Flow Determination: A study on monkeys with 15 and 35  $\mu\text{m}$  spheres
- 86 Frank, E., K. Gustvik and H. Sommerschlägl  
Cholinergic Receptors at Denervated Mammalian Motor End-Plates
- 77 Blix, A. S., E. B. Messelt and H. J. Gray  
Differential Effects of Temperature on Activity of LDH from Seal and Sheep Spleen
- 83 Henriksen, O. and T. Alner  
Effect of Spinal Sympathetic Blockade upon Local Regulation of Blood Flow in Subcutaneous Tissue

## Editorial Board

F Buchthal  
København

K. Hartala  
Turku

J Jansen  
Oslo

Y Zotterman  
Stockholm

U S von Euler  
(Editor) Stockholm

## Editorial office

Acta Physiologica Scandinavica  
Karolinska Institutet  
S-104 01 Stockholm

OR 1000  
SM  
27991  
8/1/04  
Subs  
FILIG LIBRARY  
COLLEGE

The "Acta physiologica scandinavica" are published for the Scandinavian authors and contain contributions to Physiology, Medical Chemistry or Pharmacology by Scandinavian authors or from Scandinavian laboratories. The articles are published in English, French or German. Each number consists of about 9 printed sheets, 4 numbers forming a volume. Not less than 3 volumes will appear each year. Subscriptions should be mailed to Acta Physiologica Scandinavica, Karolinska Institutet, S-104 01 Stockholm, Sweden.  
Price per volume 98 Sw. Cr.

Manuscripts from Denmark should be sent to Professor F. Buchthal,  
Universitetets Neurofysiologiske Institut, Juliane Mariesvej 35, DK-2100 København Ø.

Manuscripts from Finland should be sent to Professor K. Hartala,  
Fysiologiska Institutionen, Universitetet, Turku.

Manuscripts from Norway should be sent to Dr. med. Jan Jansen,  
Fysiologisk Institutt, Universitetet, Karl Johans Gate 47, Oslo, and

Manuscripts from Sweden should be sent to Professor Y. Zotterman,  
Wenner-Gren Center, Konferenssekreteriatet, 23 tr. Sveavägen 166, S-113 48 Stockholm.

Wyllie, L. and J. Broadin

Effect of Norepinephrine and Dopamine on Neuronal Receptors Mediating Negative Feedback Control of Norepinephrine Secretion in Human Vasoconstrictor Nerves

Farag, J., L. Bjertness and A. Hauge

The Pulmonary Vasoconstrictor Response to Hypoxia: Effects of Inhibitors of Prostaglandin Synthesis

Jacheco, F. and B. Andersson

Recovery of ADH Activity in the Urine of Goats under Normal and Stimulated Conditions

Krørup, H., J. A. Larsen and A. Mørch

Inhibition of Glucagon Effects on Splanchnic Hemodynamics and Liver Function by N, 2'-O-Dibutyryl 3',5'-Cyclic AMP (DBcAMP) in Cats

Hemminki, E.

Accumulation of Calcium by Retinal outer Segments

Fähren, P.-E. and J. Kewenter

Reflexogenic Contraction of the Ileo-Cecal Sphincter in the Cat Following Small or Large intestinal Distension

Bergendorff, A.

Intracellular Distribution of Amines Taken up by Rat Mast Cells *In Vitro*

† Commencement

Meenard, A., P. Hedqvist and M. Bygdeman

Norepinephrine Release Following Nerve Stimulation and Its Modification by Prostaglandin  $E_2$  in Human and Rabbit Oviduct

Supplement appended



## Instructions to authors

Manuscripts (2 copies) should be sent to the National Editor in double spacing on one side of paper also 21×30 cm (A 4) with 4 cm margin. A short title (max. 40 letters) may be suggested. An abstract not exceeding 200 words should be submitted.

In general a succinct style and restriction to the necessary of documentation and diagrams will effectively aids in reducing publication time.

References should be given with full title and name of journals abbreviated in accordance with the 1st Ed. of *World List of Scientific Periodicals* with volume number and first and last page.

Figures should not be larger than manuscript pages and sent in as glossy prints in a size that required for reproduction. Lettering should be large enough to permit suitable reduction preferably of uniform size. When possible diagrams and photomicrographs should extend horizontally rather than vertically in order to save space. Photomicrographs should be calibrated on (not as enlargement factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum both in number and size with text above the table (on separate sheets). Single numbers in a series should be replaced by mean and S.D. or median in the latter case with number of observations.

Key words (5-10) are recommended in order to facilitate indexing.

For abbreviations, units and symbols see special list in the Journal and recent articles.

More detailed instructions to authors are under preparation pending the recommendations of the Scandinavian Publications Committee.

## The International system of units (SI)

The following symbols and units recommended by the SI are being used in *Acta Physica Scandinavica*. Certain units not included in SI will still be permitted.

### SI units with recommended symbols

Unit	Symbol
kilogramme	kg
second millisecond	s ms
mole millimole micromole	mol mmol $\mu$ mol
picomole	nmol pmol
meter millimeter	m mm
micrometer	$\mu$ m
nanometer	nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s <sup>-1</sup> )
newton (force)	N (kg m/s <sup>2</sup> )
pascal (pressure)	Pa (N/m <sup>2</sup> )
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m <sup>2</sup> )

### Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	kl
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg
(millibar)	(1.333 bar)
curie	mbar (100 Pa)*
liter milliliter microliter	Cl
degree Celsius	l ml $\mu$ l
	°C

Conversion factors to be given in Method

# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 95

NUMBER 2

October 1975



## Contents

- 145 Kelläke, J. T. Pyörälä, P. Lehtovirta and A. Rikonen  
Cardiopulmonary Blood Volumes at Rest and during Muscular Exercise Measured by  
 $^{125}\text{I}$  in Radio-cardiography
- 153 Esseen, B., E. Jansson, J. Henriksson, A. W. Taylor and B. Salti  
Metabolic Characteristics of Fibre Types in Human Skeletal Muscle
- 165 Hansson, C. and J.-O. Kellerth  
The Postnatal Development of Some Twitch and Fatigue Properties of the Ankle  
Flexor and Extensor Muscles of the Cat
- 179 Öberg, B., R. Little, B. Rippe and B. Folkow  
The Influence of Rheological and Collapse Factors on Pre- and Post-Capillary  
Flow Resistances in the Skeletal Muscle Vascular Bed of the Cat
- 191 Kåitiskog, Ö., L.-O. Lindborn, H. R. Ulfendahl and M. Wolgast  
Kinetics of the Glomerular Ultrafiltration in the Rat Kidney: A Theoretical Study

## Short Communications

- 201 Davies, M., B. Ekblom, U. Bergh and L. L. Kanstrup-Jensen  
The Effects of Hypothermia on Submaximal and Maximal Work Performance
- 203 Andersen, P.  
Capillary Density in Skeletal Muscle of Man
- 208 Carlsson, Ch., M. Hägglund and B. K. Siesjö  
Increase in Cerebral Oxygen Uptake and Blood Flow in Immobilization Stress

Abstracts from Meeting of the Scandinavian Physiological Society  
Copenhagen 25-28 April 1975

## Communications

- 1 A. Wennmalm, A. and A. Kjellberg, Prostaglandins and Post-ischemic Muscular Vasodilation
- 2 A. Hägglund, H. T. Järvelä and P. Lehtinen, Recordings of Single Unit Activity  
from Cutaneous Nerve Fibres in Man
- Brown, M. C., J. K. S. Jensen and D. Van Essen, A Large-scale Reduction  
in Muscular Blood Flow during Postnatal Development

## Instructions to authors

Manuscripts (7 copies) should be sent to the National Editor in double spacing on one side of paper size 21×30 cm (A 4) with 4 cm margin. A short title (max. 40 letters) may be suggested. An abstract not exceeding 200 words should be submitted.

In general, a succinct style and restriction to the necessary of documentation and data effectively aids in reducing publication time.

References should be given with full title and name of journals abbreviated in accordance with *Ed. of World List of Scientific Periodicals* with volume number and first and last page number.

Figures should not be larger than manuscript pages and sent in as glossy prints in a size large that required for reproduction. Lettering should be large enough to permit suitable reduction, preferably of uniform size. When possible, diagrams and photomicrographs should extend horizontally rather than vertically in order to save space. Photomicrographs should be calibrated on the (not as enlargement factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum both in number and size, with text above the table (on separate sheets). Single numbers in a series should be replaced by mean and S.D. or mean and in the latter case with number of observations.

Key words (5–10) are recommended in order to facilitate indexing.

For abbreviations, units, and symbols see special list in the Journal and recent articles.

More detailed instructions to authors are under preparation, pending the recommendations of the Scandinavian Publications Committee.

## The international system of units (SI)

The following symbols and units recommended by the SI are being used in *Acta Physica Scandinavica*. Certain units not included in SI will still be permitted.

### SI units with recommended symbols

Units	Symbols
kilogramme	kg
second, millisecond	s, ms
mole, millimole, micromole, nanomole, picomole	mol, mmol, $\mu$ mol, nmol, pmol
meter, millimeter	m, mm
micrometer, nanometer	$\mu$ m, nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s <sup>-1</sup> )
newton (force)	N (kg m/s <sup>2</sup> )
pascal (pressure)	Pa (N/m <sup>2</sup> )
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m <sup>2</sup> )

### Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter) (calorie)	M, cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 Pa)*
curie	Ci
liter, milliliter, microliter	l, ml, $\mu$ l
degree Celsius	°C

Conversion factors to be given in Methods.

# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 95

NUMBER 2

October 1975

## Contents



- 145 Kallås, J. T. Pyörälä, P. Lehtovirta and A. Räsänen  
Cardiopulmonary Blood Volumes at Rest and during Muscular Exercise Measured by  
 $^{125}\text{I}$  in Radiocardiography

- 153 Eade, B., E. Jackson, J. Henriksen, A. W. Taylor and B. Sahlin  
Metabolic Characteristics of Fibre Types in Human Skeletal Muscle

- 158 Hamnerberg, C. and J.-O. Kellnerth

The Postnatal Development of Some Twitch and Fatigue Properties of the Aaile  
Flexor and Extensor Muscles of the Cat

- 178 Öberg, B., R. Little, B. Rippe and B. Folkow

The Influence of Rheological and Collapse Factors on Pre- and Post-Capillary  
Flow Resistances in the Skeletal Muscle Vascular Bed of the Cat

- 191 Kildskog, O., L. O. Lindbom, H. R. Ulfendahl and M. Wolgast

Kinetics of the Glomerular Ultrafiltration in the Rat Kidney: A Theoretical Study

## Short Communications

- 201 Davies, M. B., Ekblom, U., Bergh and L.-L. Kanstrup-Jensen

The Effects of Hypothermia on Submaximal and Maximal Work Performance

- 203 Andersen, P.

Capillary Density in Skeletal Muscle of Man

- 206 Carlsson, Ch., M. Hägerdal and B. K. Stenjö

Increase in Cerebral Oxygen Uptake and Blood Flow in Immobilization Stress

Abstracts from Meeting of the Scandinavian Physiological Society  
Copenhagen 25-28 April 1975

## Communications

- 1 A. Wennberg, A. and J. Kihlborn, Prostaglandins and Post-ischemic Muscular Vaso-  
dilation

- 2 A. Häkkinen, H. T. Järvelä and P. Laurinen, Recordings of Single Unit Activity  
from Cat Spinal Nerve Fibers in Man

# Editorial Board

F Buchthal  
København

K. Harttala  
Turku

J Jansen  
Oslo

Y Zotterman  
Stockholm

U S von Euler  
(Editor) Stockholm

## Editorial office

Acta Physiologica Scandinavica  
Karolinska Institutet  
S 104 01 Stockholm

The "Acta physiologica scandinavica" are published for the Scandinavian Society for Physiology and contain contributions to Physiology Medical Chemistry or Pharmacology by Scandinavian authors or from Scandinavian laboratories. The articles are published in English, French or German. Each number consists of about 8 printed sheets, 4 numbers forming a volume. Not more than 3 volumes will appear each year. Subscriptions should be mailed to Acta Physiologica Scandinavica, Karolinska Institutet, S-104 01 Stockholm, Sweden.  
Price per volume 98 Sw Cr

Manuscripts from Denmark should be sent to Professor F. Buchthal  
Universitetets Neurofysiologiske Institut, Julevej 36 Dk 2100 København Ø

Manuscripts from Finland should be sent to Professor K. Harttala,  
Fysiologiska Institutionen, Universitetet, Turku.

Manuscripts from Norway should be sent to Dr. med. Jan Jansen  
Fysiologisk Institutt, Universitetet Karl Johans Gate 47 Oslo and

Manuscripts from Sweden should be sent to Professor Y. Zotterman,  
Wenner-Gren Center Konferenssekretariatet, 23 tr Sveavägen 186 113 48 Stockholm

- 
- 4 A Russel J. T. and N. A. Thorn: Adenosine-Triphosphate Induced Emptying of Isolated Bovine Neurohypophyseal Secretory Granule Contents
  - 5 A Hillensjö T.: Hormonal Control of Glycolysis and Oocyte Maturation in the Preovulatory Rat Ovarian Follicle
  - 6 A Haugen P. and O. Sten-Knudsen: Early Mechanical Response in Isolated Frog Skeletal Muscle Fibres
  - 7 A Jodal M. and O. Lundgren: Demonstration of Tissue Hyperosmolality in the Tip of Intestinal Villi during Sodium Chloride Absorption
  - 8 A Järhult J.: Osmolar Control of Extracellular and Plasma Volume in Hemorrhagic Hypotension
  - 9 A Hoppe P. P., K. Johansen G. M. O. Maloiy and V. Musewe: Thermal Panting Reduces Oxygen Uptake in the DB/DB<sub>1</sub> *Rhynchotegus kirkii*
  - 10 A Kornum L. O., L. Bjørnsen P. Krag C. H. Nielsen and P. E. Paulsen: Tensile Strength of Gas Saturated Biological Liquids Exposed to Successive High Pressures
  - 11 A Flemström G.: Ion Transport in Non-acid Secreting Gastric Epithelium
  - 12 A Wennergren G.: Aspects of the Central Interaction between Excitatory Chemoreceptor and Inhibitory Baroreceptor and Cardiac Receptor Influences
  - 13 A Hermansen L. O. Væge C. Williams A. Helland and L. Nilssen: Glycogen Synthesis and Lactate Metabolism during Recovery after Maximal Exercise in Man
  - 14 A Hallinen M. O. M. O. K. Hakumäki and H. S. S. Barajas: Initial Effects of Endotoxin on Autonomic Efferent Discharges in Dogs
  - 15 A Sollevi A., P. Hjemdahl and B. B. Fredholm: Vascular and Metabolic Effects of Local Cooling in Canine Subcutaneous Adipose Tissue
  - 16 A Euler C. von, I. Marttila J. Remmers and T. Trippenbach: On the Functional Significance of the so-called "Pneumotoxic Centre" for the Respiratory Pattern
  - 16 A Barallindh T.: Isolated Gastric Glands: A New Approach for the Study of Gastric Secretion

- 7 A Vaage, J. The Role of Parasympathetic Bronchomotor Fibres in Airway Constriction after Sudden Intravascular Platelet Aggregation
- 8 A Sjöfåre, L. Adrenoceptor Mediated Positive and Negative Feedback Control of Noradrenaline Secretion from Human Vasoconstrictor Nerves
- 10 A Berg, T. and J.-G. Iversen, Hormone Stimulated K<sup>+</sup> Transport in Isolated Rat Liver Cells
- 10 A Liang, B., R. Shvartsman, M. Halbläck and B. Folkow. Reduced Muscle Efficiency during Vibration-Induced Adaptation of Contractile Force in Cat Solenoid Muscle
- 11 A Grant, G. S. Lundgren and H. Sjöfåre, Columnar Distribution of U-Fibres from the Postcricoid Cerebral Projection Area of the Cat's Group 1 Muscle Affects
- 12 A Pehr, T. S. L. Nielsen and N. A. Lassen, Water Permeability in the Human Forearm during Graded Exercise
- A Weber, R. E., Environmental Adaptation and Multiplicity in Hemoglobins of the Pacific Plaice and Flounder
- A Pasaka, W. P. and O. Henriksen, Vascular Resistance in Peripheral Blood Vessels at Normotension and at Local Orthostatic Hypertension in Healthy Humans
- 1 A Rasmussen, S. N. Effects of Androgenic Hormone on the Intramural Distribution of Red Cells and Plasma in the Rat
- 1 A Colding-Jørgensen, M., Two Types of Adaptation in Snail Neurons
- 7 A Krogh, L. M. Transient "Hypertrophy" of the Anterior Tibial Muscle after Denervation Combined with Stretch

#### Demonstrations

- 19 A Borg, J. Van der J. M. Halbertsma, F. O. A. Van der Meché and S. Müller. Coordination of the Limbs during Locomotion in the Cat: Basic "Programs" of Movement
- 20 A Pasaka, W. P. and P. Beijnen, Application of a Process Computer (Wang 800) in Experimental Physiology
- 21 A Rasmussen, S. N., Exchange Kinetics of Extravascular Albumin in the Rat Kidney Studied by Means of a Sharp Input Tracer Technique
- 22 A Henriksen, O., Estrous Cycle of Zinc-Deficient Female Rats
- 22 A Henriksen, O. Effect of Local Vascular Stasis on Blood Flow in Human Subcutaneous Tissue
- 23 A Colding-Jørgensen, M., Stimulating Adaptation of Excitable Cells on Analog Computers
- 24 A Sjöfåre, L. and W. Grampp, Membrane Noise in Receptor Neurons
- 25 A Plesner, H. Estimation of Pulmonary Arterial Compliance from Registrations of Oscillating Blood Flow and Pressure
- 26 A Abrahamsson, T. and S. Nilsson, Phenylethanolamine-N-methyl Transferase (PNMT) Activity in Sympathetic Ganglia of the Dog
- A Nilsson, S., S. Holmgren and D. J. Grove, Autonomic Nerve Control of the Dog's Spleen
- A Flope, R., S. Holmgren and S. Nilsson, Drug Effects on Gas Production and Sino Muscles in Fish Swimbladder
- A Sjöfåre, L. A Machine for On Line Calculation of Time Interval and Peak Amplitude Histograms
- 1 A Engberg, L., J. A. Fleitman, K. Kadzifajew and J. O. G. Lambert, Actions of Local Anesthetics Applied by Microiontophoresis to Motoneurons
- 1 A Jodal, M., D. A. Halbläck, J. Svanvik and O. Lundgren, A Photomicrographic Method for Continuous Determination of Net Water Transport across the Intestinal Epithelium *in vivo*
- 2 A Ahlqvist, G., Anaerobic Glycogen Metabolism in the Amputated Snail *Marisa cornu*
- 2 A Björk, J. and J. Mathsen, Correction for Changes in Counting Efficiency during Duration of  $^{45}\text{Ca}$  from Subcutaneous Fat
- 4 A Folke, M. and M. Petäaho, The Effect of Alanine on Cell Membrane Potentials in Rat Liver
- 14 A Frenning, B., The Disappearance of Hydrogen Ions from the Normal Human

- 48 A Hultborn R and T Lichtneckert, Reactant Introduction in the Course of Assay of Oxygen Consumption Performed by Microspectrophotometry
- 49 A Vinten J and J Giffmann Self Exchange of 3-O Methylglucose in Isolated Fat Concentration dependence and effect of Insulin
- 50 A Jalling O and C. Olsen The Influence of pH on the Lactate Production by Isolated Parenchymal Rat Liver Cells
- 51 A Dalby B E Engberg J T Russell and N A. Thorn Release of a Large Fraction of Oxytocin Stored in Rat Neurohypophyses by Intensive Stimulation *in vitro*
- 52 A Kierup N J A. Larsen and A Munck, Hepatosplanchnic Hemodynamics and Liver Function during Graded Hypoxia
- 53 A Khan I B Rosberg K Herlitz and K Åhrén, Cyclic AMP Content and Phosphodiesterase Activity in Rat Corpora Lutea of Different Ages
- 55 A Gustafsson, L. and P. Hedqvist, Prostaglandin Formation Participates in the Control of Tone and Contractility in the Iris Sphincter Muscle
- 56 A Secher N H J P Clausen K Klausen, I Noer and J Trap-Jensen, Total and Regional Oxygen Uptakes, Blood Flows and Arterial Pressures during Exercise with Smaller and Greater Muscle Groups
- 57 A Guild C Cortical Magnification Factor in the Foveal Region of Vervet Monkey
- 57 A Paloheimo M and M Folke, Hyperpolarization of the Cell Membrane in Rat Liver after Partial Hepatectomy
- 58 A Grillner S., S Rosagnol and P Wallén Phase Dependent Reflex Reversal during Swimming in the Spinal Dogfish
- 60 A Valio H and M G Parkki Deterioration of Drug Biotransformation in Rat Liver by a Fungicide Dichloro
- 61 A Marniemi J H Valio and M G Parkki Conjugative Biotransformation of Xenobiotics in the Gunn Rat
- 62 A Christensen, N J J Trap-Jensen J P Clausen I Noer A. R Krosgaard and O A Larsen Effect of Beta receptor Blockade on Heart Rate, Hepatic Blood Flow and Circulating Norepinephrine during Exercise in Man
- 63 A Mertz, J P E. Paulev and S B Pedersen Breathing at Supramaximal Ventilations during Rebreathing and Steady State Exercise
- 65 A Hellstrand P Oxygen Sensitivity of Spontaneous and Induced Contractions in Venous Smooth Muscle
- 66 A Vilhardt, H and M Holgate Heat Inactivation of Bovine Neurophysins
- 67 A Brendsted H E. and E. Westergaard Vesicular Transport of Proteins from Blood to Brain during Acute Hypertension
- 68 A Karimark, B and M Sothell, An Electrode for Micropuncture Measurements of Pot
- 69 A Lagercrantz, H G Fried and T Hökfelt, Estimation of the Norepinephrine Content in Sympathetic Nerve Terminal
- 70 A Järvelähti M and J Moring Summation of Receptor Potentials in the Retina of the Fly *Calliphora* Studied by Very Short Double Flashes
- 71 A Tjernshaugen H E. Haug and K M Gautvik Regulation of Cell Growth and Prolactin Production in Cultured Rat Pituitary Cells
- 72 A Fredriksson, A and U Böderberg Persistent Effects of Large Doses of Electrolytes in the Food of Lactating Rats on the Kidney Function of their Offspring
- 73 A Bolstad O and A. Ersland Energy Metabolism in Different Human Skeletal Muscles during Voluntary Isometric Contraction
- 74 A Rosenkilde P Metabolism of Iodine and Iodinated Thyroid Products in Amphibia
- 75 A Johansson H and H Sjöfvenius, Location of Neurones in the Cat *Mesencephalon* that are Activated by Hind Limb Group I Muscle—and by Skin Afferents
- 76 A Albertsson Wiklund K. and O Isaksson, Sensitivity to Growth Hormone of Diaphragm Muscle from Young Normal Rats
- 77 A Pedersen, S S M Grundstein, J Mertz, J Millic Emil and P E. Paulev Tree-component Output Analysis of Respiration during Rebreathing and Exercise in
- 79 A Källskog O L O Lindbom H R Ulfendahl and M *Physiology of*

No Supplement appended

Printed by C. G. Gundersen for the Norwegian Scientific Committee

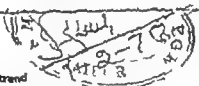
# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 95

NUMBER 3

November 1975

## Contents

- 
- 208 Östman, L. and N.-O. Sjöstrand  
Reduced Urinary Norepinephrine Excretion during Rest, Exercise and Cold Stress in Trained Rats: Comparison between Physically Trained Rats, Cold-Acclimated Rats and Warm-Acclimated Rats
- 218 Nilsson, B., G. Holmgren and D. J. Grove  
Effects of Drugs and Nerve Stimulation on the Spleen and Arteries of Two Species of Dog Fish, *Squalius laietanus* and *Squalus acanthias*
- 227 Hagenberg, C. and J.-O. Kellerth  
Studies of Some Twitch and Fatigue Properties of Different Motor Unit Types in the Ankle Muscles of the Adult Cat
- 237 Hagenberg, C. and J.-O. Kellerth  
The Postnatal Development of Some Twitch and Fatigue Properties of Single Motor Units in the Ankle Muscles of the Kitten
- 247 Nilsson, B.  
Effect of Buffer Acidification on Gastro Acid Responses to Urethane in Pavlov Pouch Dogs
- 257 Sigurdsson, B. B., B. Uvellöf and B. Johansson  
Relative Contribution of Superficially Bound and Extracellular Calcium to Activation of Contraction in Isolated Rat Portal Vein
- 267 Josefsson, J., G. Johansson and B. E. Hansson  
Inhibition of Induced Phagocytosis in Amoeba proteus by Membrane Stabilizing Drugs
- 277 Hellekær, G. and V. Gjesdal  
Depression of Tact Responses by Local or Intravascular Administration of Salicylates in the Rat
- 287 Kålleberg, O., L.-O. Undheim, H. R. Ulfendahl and M. Wolgast  
Kinetics of the Glomerular Ultrafiltration in the Rat Kidney: An Experimental Study
- 297 Norberg, K., B. Gustafsson and B. K. Siesjö  
Effects of Hypoxia of 10-45 Seconds Duration on Energy Metabolism in the Cerebral Cortex of Unanesthetized and Anesthetized Rats
- 307 Kravitz, N.  
Effects of Histamine, Vasopressin, and Angiotensin II on Hepaticoplacental Hemodynamics, Liver Function, and Hepatic Metabolism in Cats

Continued on back cover



- 48 A Hultborn R and T Lichtneckert, Reactant Introduction In the Course of Assay of Oxygen Consumption Performed by Microspectrophotometry
- 49 A Vinten J and J Gillemann Self Exchange of 3-O-Methylglucose in Isolated Fat Cells. Concentration dependence and effect of Insulin
- 50 A Jølling Ø and C Olsen The Influence of pH on the Lactate Production by Isolated Parenchymal Rat Liver Cells
- 51 A Dalby S E Engberg J T Russell and N A Thorn Release of a Large Fraction of Oxytocin Stored in Rat Neurohypophyses by Intensive Stimulation *in vitro*
- 52 A Krarup N J A. Larsen and A. Munck, Hepatosplanchnic Hemodynamics and Liver Function during Graded Hypoxia
- 53 A Khan I S Rosberg K Herlitz and K. Åhrén Cyclic AMP Content and Phosphodiesterase Activity in Rat Corpora Lutea of Different Ages
- 55 A Gustafsson L and P Hedqvist, Prostaglandin Formation Participates in the Control of Tone and Contractility in the Iris Sphincter Muscle
- 56 A Secher N H., J P Clausen K. Klausen I Noer and J Trap-Jensen, Total and Regional Oxygen Uptakes, Blood Flows and Arterial Pressures during Exercise with Smaller and Greater Muscle Groups
- 57 A Guild C Cortical Magnification Factor in the Foveal Region of Vervet Monkey
- 57 A Paloheimo M and M Folke Hyperpolarization of the Cell Membrane in Rat Liver after Partial Hepatectomy
- 58 A Grillner S S Rossignol and P Wallén Phase Dependent Reflex Reversal during Swimming in the Spinal Dogfish
- 60 A Valio H and M G Parkki Deterioration of Drug Biotransformation in Rat Liver by a Fungicide Dichloro
- 61 A Marniemi J H Valio and M G Parkki Conjugative Biotransformation of Xenobiotics in the Gunn Rat
- 62 A Christensen N J J Trap-Jensen J P Clausen, I Noer A. II Krogsgaard and Ø A Larsen Effect of Beta receptor Blockade on Heart Rate Hepatic Blood Flow and Circulating Norepinephrine during Exercise in Man
- 63 A Mertz J P E. Paulev and S S Pedersen, Breathing at Supramaximal Ventilations during Rebreathing and Steady State Exercise
- 65 A Hellstrand P Oxygen Sensitivity of Spontaneous and Induced Contractions in Venous Smooth Muscle
- 66 A Vilhardt, H and M Holgate Heat Inactivation of Bovine Neuropeptides
- 67 A Brandsted H II and E. Westergaard Vesicular Transport of Proteins from Blood to Brain during Acute Hypertension
- 68 A Karlmark, B and M Sothell An Electrode for Micropuncture Measurements of  $P_{CO_2}$
- 69 A Lagercrantz, H., G Fried and T Håkfelt, Estimation of the Norepinephrine Content in Sympathetic Nerve Terminal
- 70 A Järvilehto M and J Moring Summation of Receptor Potentials in the Retina of the Fly *Calliphora* Studied by Very Short Double Flashes
- 71 A Tjernshaugen H E. Haug and K M Gautvik, Regulation of Cell Growth and Prolactin Production in Cultured Rat Pituitary Cells
- 72 A Fredriksson, A. and U Söderberg Persistent Effects of Large Doses of Electrolytes in the Food of Lactating Rats on the Kidney Function of their Offspring
- 73 A Bolstad G and A. Erland Energy Metabolism in Different Human Skeletal Muscles during Voluntary Isometric Contraction
- 74 A Rosenkilde P., Metabolism of Iodine and Iodinated Thyroid Products in Amphibia
- 75 A Johansson, H and H Siltvenius, Location of Neurones in the Cat *Medulla oblongata* that are Activated by Hind Limb Group I Muscle—and by Skin Afferents
- 76 A Albertsson Wiklund K and O Isaksson, Sensitivity to Growth Hormone of Diaphragm Muscle from Young Normal Rats
- 77 A Pedersen S S., M Grundstein J Mertz, J Milic-Emili and P E. Paulev Tree-component Output Analysis of Respiration during Rebreathing and Exercise in Man
- 79 A Källskog Ö., L O Lindbom H R Ulfendahl and J. H. Larsson Glomerular Ultrafiltration

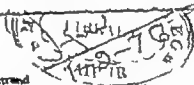
# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 95

NUMBER 3

November 1975

## Contents



- 209 Ostenson, L. and H. O. Sjögstrand  
Reduced Urinary Noradrenaline Excretion during Rest, Exercise and Cold Stress in Trained Rats: A Comparison between Physically Trained Rats, Cold Acclimated Rats and Warm-Acclimated Rats
- 219 Nilsson, S., S. Holmgren and D. J. Grove  
Effects of Drugs and Nerve Stimulation on the Spleen and Arteries of Two Species of Dog  
*Bath, Scyllorhina carinata* and *Squalus acanthias*
- 231 Hansson, C. and J. O. Kellerth  
Studies of Some Twitch and Fatigue Properties of Different Motor Unit Types in the Ankle Muscles of the Adult Cat
- 243 Hansson, C. and J.-O. Kellerth  
The Postnatal Development of Some Twitch and Fatigue Properties of Single Motor Units in the Ankle Muscles of the Kitten
- 253 Nilsson, G.  
Effect of Buffer Acidification on Gastric Acid Responses to Urecholes in Pavlov Pouch Dogs
- 263 Sigurdsson, S. B., B. Uredal and B. Johansson  
Relative Contribution of Superficially Bound and Extracellular Calcium to Activation of Contraction in Isolated Rat Portal Vein
- 270 Jönasson, J.-O., G. Johansson and S. E. Hansson  
Inhibition of Induced Phagocytosis in Amoeba proteus by Membrane Stabilizing Drugs
- 283 Hellekant, G. and V. Gopal  
Depression of Taste Responses by Local or Intravenous Administration of Salicylates in the Rat
- 293 Kihlström, Ö., L. Lindbom, H. R. Ulfendahl and M. Wolgast  
Kinetics of the Glomerular Ultrafiltration in the Rat Kidney: An Experimental Study
- 301 Norberg, K., B. Oulertoff and B. J. Sjöberg  
Effects of Hypoxia of 10-45 Seconds Duration on Energy Metabolism in the Cerebral Cortex of Unanesthetized and Anesthetized Rats
- 311 Krumm, M.  
Effects of Histamine, Vasopressin, and Angiotensin II on Hepatoportal Hemodynamics, Liver Function, and Hepatic Metabolism in Cats

- 48 A Hultborn R and T Lichtneckert, Reactant Introduction in the Course of Assay of Oxygen Consumption Performed by Microspectrophotometry
- 49 A Vinten J and J Gillemann, Self Exchange of 3 O Methylglucose in Isolated Fat Cells. Concentration dependence and effect of Insulin
- 50 A Jølling O and C Olsen The Influence of pH on the Lactate Production by Isolated Parenchymal Rat Liver Cells
- 51 A Dalby E, E. Engberg J T Russell and N A. Thorn Release of a Large Fraction of Oxytocin Stored in Rat Neurohypophyses by Intensive Stimulation *in vitro*
- 52 A Krarup N, J A. Larsen and A. Munck, Hepatoportal Hemodynamics and Liver Function during Graded Hypoxia
- 53 A Khan I S Rosberg K Herlitz and E. Åhrén, Cyclic AMP Content and Phosphodiesterase Activity in Rat Corpora Lutea of Different Ages
- 55 A Gustafsson L and B Hedqvist, Prostaglandin Formation Participates in the Control of Tone and Contractility in the Iris Sphincter Muscle
- 58 A Secher N H J P Clausen K Klausen I Noer and J Trap-Jensen Total and Regional Oxygen Uptakes, Blood Flows and Arterial Pressures during Exercise with Smaller and Greater Muscle Groups
- 57 A Guld C, Cortical Magnification Factor in the Foveal Region of Vervet Monkey
- 57 A Paloheimo M and M Folke Hyperpolarization of the Cell Membrane in Rat Liver after Partial Hepatectomy
- 58 A Grillner S B Rossignol and P Wallén, Phase Dependent Reflex Reversal during Swimming in the Spinal Dogfish
- 60 A Vainio H and M G Parkki Deterioration of Drug Biotransformation in Rat Liver by a Fungicide Dichloro
- 61 A Marniemi J H Vainio and M G Parkki Conjugative Biotransformation of Xenobiotics in the Gunn Rat
- 62 A Christensen, N J., J Trap-Jensen J P Clausen, I Noer A. R Krogsgaard and O A. Larsen Effect of Beta receptor Blockade on Heart Rate Hepatic Blood Flow and Circulating Norepinephrine during Exercise in Man
- 63 A Mertz, J P E Paulev and S S Pedersen, Breathing at Supramaximal Ventilations during Rebreathing and Steady State Exercise
- 65 A Hellstrand P Oxygen Sensitivity of Spontaneous and Induced Contractions in Vascular Smooth Muscle
- 66 A Vilhardt, H and M Holgate Heat Inactivation of Bovine Neurophysins
- 67 A Brandstad H E. and E. Westergaard Vesicular Transport of Proteins from Blood to Brain during Acute Hypertension
- 68 A Karlmark B and M Sothell An Electrode for Micropuncture Measurements of  $P_{CO_2}$
- 69 A Lagercrantz, H G Fried and T Hökfelt, Estimation of the Norepinephrine Content in Sympathetic Nerve Terminal
- 70 A Järvilehto M and J Moring Summation of Receptor Potentials in the Retina of the Fly *Cattophora* Studied by Very Short Double Flashes
- 71 A Tjernsæugen, H E. Heug and K M Gautvik, Regulation of Cell Growth and Prolactin Production in Cultured Rat Pituitary Cells
- 72 A Fredriksson, A. and U Söderberg Persistent Effects of Large Doses of Electrolytes in the Food of Lactating Rats on the Kidney Function of their Offspring
- 73 A Bolstad G and A. Ersland Energy Metabolism in Different Human Skeletal Muscles during Voluntary Isometric Contraction
- 74 A Rosenkilde P., Metabolism of Iodine and Iodinated Thyroid Products in Amphibia
- 76 A Johansson, H and H Sjöfvenius, Location of Neurons in the Cat *Medulla oblongata* that are Activated by Hind Limb Group I Muscle—and by Skin Afferents
- 78 A Albertsson Wiklund K and O Isaksson, Sensitivity to Growth Hormone of Diaphragm Muscle from Young Normal Rats
- 77 A Pedersen, S S., M Grundstein J Mertz, J Milio Emilii and P E. Paulev Tree-component Output Analysis of Respiration during Rebreathing and Exercise in Man
- 79 A Källskog O., L O Lindbom, H R Ulfendahl and M Wolgast, Kinetics of the Glomerular Ultrafiltration

# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 96

NUMBER 4

December 1976

## Contents

- 353 Helander H F E. R. Bendert, L. L. Shanbour and W. S. Reine  
Reversibility of Effects of Very Hypotonic Fluids on *In Vitro* Frog Gastro Muscles: A Functional and Morphological Study
- 364 Kjellberg O. and M. Wolgerst  
Effects of Elevated Interstitial Pressure on the Renal Cortical Hemodynamics
- 373 Pyörälä C., E. Blaesjö, U. Bergqvist and B. Uvnäs  
On the Possible Involvement of Sulphomucopolysaccharides in the Storage of Catecholamines within the Central Nervous System
- 383 Linder B. and G. Chisolin  
The Interstitial Space of Adipose Tissue as Determined by Single Injection and Equilibrium Techniques
- 391 Harri M. M. E. and J. Valtola  
Comparison of the Effects of Physical Exercise, Cold Acclimation and Repeated Injections of Isoprenaline on Rat Muscle Enzymes
- 400 Leppäaho J. M. Rapell, R. Varti and T. Ranta  
Secretion of Anterior Pituitary Hormones in Man: Effects of Ethyl Alcohol
- 407 Decker L. and H. Tjälve  
Studies on the Renal Uptake of Vitamin D in the Mouse and the Quail
- 417 Holstein B.  
Gastric Acid Secretion in Teleostean Fish: A Method for the Continuous Collection of Gastric Effluence from Swimming Fish and Its Response to Histamine and Pentagastrin
- 424 Ujeng B. and P. H. Ilgen  
On the Mechanism of Inhibitory Action of Vibrations as Studied in Molluscan Catch Muscle and in Vertebrate Vascular Smooth Muscle
- 431 Appelberg B. T. Jönsson and H. Johansson  
Rubrospinal Control of Static and Dynamic Postural Neurons
- 441 Jodal M. -A. Hultback, J. Svamvik and M. Lundgren  
A Method for the Continuous Study of Net Water Transport in the Feline Small Bowel
- 448 Borrebaek B.  
Oxidation of Carnitine and Glycerophosphate in Suspensions of Rat Liver Mitochondria at Varying Levels of Palmitate and Coenzyme A

## Instructions to authors

Manuscripts (2 copies) should be sent to the National Editor in double spacing on one side of paper size 21×30 cm (A 4) with 4 cm margin. A short title (max. 40 letters) may be suggested. An abstract not exceeding 200 words should be submitted.

In general a succinct style and restriction to the necessary of documentation and discussion effectively aids in reducing publication time.

References should be given with full title and name of journals, abbreviated in accordance with 4th Ed. of *World List of Scientific Periodicals* with volume number and first and last page numbers.

Figures should not be larger than manuscript pages and sent in as glossy prints in a size larger than that required for reproduction. Lettering should be large enough to permit suitable reduction and preferably of uniform size. When possible diagrams and photomicrographs should extend horizontally rather than vertically in order to save space. Photomicrographs should be calibrated on the print (not as enlargement factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum both in number and size with text above the table (not on separate sheets). Single numbers in a series should be replaced by mean and S.D. or mean and S.E. in the latter case with number of observations.

Key words (5–10) are recommended in order to facilitate indexing.

For abbreviations, units and symbols see special list in the Journal and recent articles.

More detailed instructions to authors are under preparation pending the recommendations of The Scandinavian Publications Committee.

## The international system of units (SI)

The following symbols and units recommended by the SI are being used in *Acta Physiologica Scandinavica*. Certain units not included in SI will still be permitted.

### SI units with recommended symbols

Units	Symbol
kilogramme	kg
second millisecond	s ms
mole millimole micro-	
mole nanomole	mol mmol $\mu$ mol
picomole	nmol pmol
meter millimeter	
micrometer	
nanometer	m mm $\mu$ m nm
candela	cd
steradian	sr
hertz (frequency)	Hz ( $s^{-1}$ )
newton (force)	N ( $kg \cdot m/s^2$ )
pascal (pressure)	Pa ( $N/m^2$ )
joule (energy)	J ( $N \cdot m$ )
watt (effect)	W ( $J/s$ )
lumen (lightflow)	lm (cd $\cdot$ sr)
lux (illumination)	lx ( $lm/m^2$ )

### Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mer-	mm Hg
cury)	(1.333 bar)
(millibar)	mbar (100 Pa)*
curie	Ci
liter milliliter micro-	
liter	l ml $\mu$ l
degree Celsius	$^{\circ}C$

Conversion factors to be given in Methods.

# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 95

NUMBER 4

December 1975

## Contents

34-2-76

- 353 Helander, H. F. S., S. S. Saunders, L. L. Sherboor and W. S. Rehm  
Reversibility of Effects of Very Hypotonic Fluids on *in Vitro* Frog Gastric Mucosa: A Functional and Morphological Study
- 364 Käflskog, O. and M. Wolgast  
Effect of Elevated Interstitial Pressure on the Renal Cortical Hemodynamics
- 373 Proctor, C., E. Blaschke, U. Bergqvist and B. Uvnäs  
On the Possible Involvement of Sulphomucopolysaccharides in the Storage of Catecholamines within the Central Nervous System
- 383 Linde, B. and G. Chissov  
The Interstitial Space of Adipose Tissue as Determined by Single Injection and Equilibration Techniques
- 391 Harri, M. N. E. and J. Valtola  
Comparison of the Effects of Physical Exercise, Cold Acclimation and Repeated Injections of Propionine on Rat Muscle Enzymes
- 400 Leppäluoto, J., M. Rapola, R. Varti and T. Ranta  
Secretion of Anterior Pituitary Hormones in Male Effects of Ethyl Alcohol
- 407 Dencker, L. and H. Tjälve  
Studies on the Renal Uptake of Vitamin D<sub>3</sub> in the Mouse and the Quail
- 417 Holteir, B.  
Gastro Acid Secretion in Teleostean Fish: A Method for the Continuous Collection of Gastric Effluence from a Swimming Fish and its Response to Histamine and Pentagastrin
- 424 Ueno, S. and P. Hallgren  
On the Mechanism of Inhibitory Action of Vibrations as Studied in a Molluscan Catch Muscle and in Vertebrate Vascular Smooth Muscle
- 431 Appelberg, B., T. Jännekoj and H. Johansson  
Autoregulation Control of Sympathetic and Dynamic Fumarate Neurones
- 441 Jodal, M., D. A. Hellblück, J. Svanvik and G. Lundgren  
A Method for the Continuous Study of Net Water Transport in the Feline Small Bowel
- 448 Borrebaek, B.  
Acylation of Cardiolipins and Glycophospholipids in Suspensions of Rat Liver Mitochondria in Varying Levels of Palmitate and Coenzyme A

## Instructions to authors

Manuscripts (2 copies) should be sent to the National Editor in double spacing on one side of paper size 21×30 cm (A 4) with 4 cm margin. A short title (max. 40 letters) may be suggested. An abstract not exceeding 200 words should be submitted.

In general, a succinct style and restriction to the necessary of documentation and discussion effectively aids in reducing publication time.

References should be given with full title and name of journals, abbreviated in accordance with 4th Ed. of *World List of Scientific Periodicals* with volume number and first and last page numbers.

Figures should not be larger than manuscript pages and sent in as glossy prints in a size larger than that required for reproduction. Lettering should be large enough to permit suitable reduction or preferably of uniform size. When possible, diagrams and photomicrographs should extend horizontally rather than vertically in order to save space. Photomicrographs should be calibrated on the print (not as enlargement factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum both in number and size with text above the table (not on separate sheets). Single numbers in a series should be replaced by mean and S.E. or mean and S.E. in the latter case with number of observations.

Key words (5–10) are recommended in order to facilitate indexing.

For abbreviations, units and symbols see special list in the Journal and recent articles.

More detailed instructions to authors are under preparation, pending the recommendations of the Scandinavian Publications Committee.

## The international system of units (SI)

The following symbols and units recommended by the SI are being used in *Acta Physiologica Scandinavica*. Certain units not included in SI will still be permitted.

### SI units with recommended symbols

Units	Symbols
kilogramme	kg
second millisecond	s ms
mole millimole micromole	mol mmol $\mu$ mol
nanomole	nmol pmol
meter millimeter	m mm $\mu$ m $\mu$ m
micrometer	
nanometer	
candela	cd
steradian	sr
hertz (frequency)	Hz (s <sup>-1</sup> )
newton (force)	N (kg m/s <sup>2</sup> )
pascal (pressure)	Pa (N/m <sup>2</sup> )
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (lightflow)	lm (cd sr)
lux (illumination)	lx (lm/m <sup>2</sup> )

### Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N) <sup>a</sup>
(millimeters of mercur)	mm Hg
curie	(1.333 bar)
(millibar)	mbar (100 Pa)
cune	Cl
liter milliliter micro-	
liter	l ml $\mu$ l
degree Celsius	°C

Conversion factors to be given in Methods.

Isaacs, R.

Baroregulation during Static Work with the Legs

Isaacs, W. P. and O. Henriksen

Vascular Resistance in Peripheral Blood Vessels at Normotension and at Local Orthostatic Hypertension in Healthy Humans

Isaacs, J. U. Lahti, H. Lybeck, J. Partanen, T. Ranta and P. Vinkkunen

Basal Secretion of ACTH, GH, LH and TSH in Man

Isaacs, G.

Effect of Bulbar Acidification on Basal Secretion of Acid and Gastrin in Dog

Järnåker, A. and A. von der Decken

Synthesis of Brain-Specific Acidic Proteins in Rat and Mouse Cerebral Slices

† Correspondence:

Hedqvist, P. and A. Mörnved

Central Presynaptic  $\alpha$ - and  $\beta$ -Adrenoceptor Mediated Control of Noradrenaline Release in Human  
Splanchnic Nerve

† Address

† Extended Supplement:

† Pflügers Archiv 426, P. E. Pahlke. Extrinsic Nervous Control of the Ileo-occal Sphincter in the Cat



## Editorial Board

F Buchthal  
København

K. Hartela  
Turku

J Jansen  
Oslo

Y Zotterman  
Stockholm

U S von Euler  
(Editor) Stockholm

## Editorial office

Acta Physiologica Scandinavica  
Karolinska Institutet  
S-104 01 Stockholm

The "Acta physiologica scandinavica" are published for the Scandinavian Society for Physiology and contain contributions to Physiology, Medical Chemistry or Pharmacology by Scandinavian authors or from Scandinavian laboratories. The articles are published in English, French or German. Each number consists of about 9 printed sheets, 4 numbers forming a volume. Not more than 3 volumes will appear each year. Subscriptions should be mailed to Acta Physiologica Scandinavica, Karolinska Institutet, S-104 01 Stockholm, Sweden.  
Price per volume 88 Sw. Cr.

Manuscripts from Denmark should be sent to Professor F. Buchthal,  
Universitets Neurofysiologiske Institut, Juliane Mariesvej 38, DK 2100 København Ø.

Manuscripts from Finland should be sent to Professor K. Hartela,  
Fysiologiska Institutionen, Universitetet, Åbo.

Manuscripts from Norway should be sent to Dr. med. Jan Jansen,  
Fysiologisk Institutt, Universitetet Karl Johans Gate 47, Oslo, and

Manuscripts from Sweden should be sent to Professor Y. Zotterman,  
Wenner Gren Center Konferenssekreterariatet, 23 tr.: Sveavägen 188, 113 48 Stockholm.

1975, 95, 15  
 Läkemedel AB, Södertälje, Sweden  
 or, AB Draco, Lund, Sweden

## lin and <sup>3</sup>H Terbutaline tized Rat

ONE and ÅKE RYRHELD

ber 1974

The biliary excretion of H-benflin and H  
 tyridol. scand. 1975 95 15

and bile from unanesthetized rats with mo-  
 nokinetic data concerning intake (0.2 µmol kg<sup>-1</sup>)  
 on are presented. The following values for intake  
 ml min<sup>-1</sup> kg<sup>-1</sup> and bile clearance 0.03-0.04 ml  
 in from the literature. For terbutaline the corre-  
 0.12-0.98 ml min<sup>-1</sup> kg<sup>-1</sup> respectively

rats anesthetized bile fistula animals have  
 peration may effect the excretory function  
 rats, which have been allowed to recover  
 tage. Various techniques to obtain interrup-  
 tion of the enterohepatic cycle have been  
 al point of view an animal model without  
 referred (Light *et al* 1959). The knowledge  
 g unanesthetized rats is scanty in the pre-  
 n and terbutaline have been studied in  
 enterohepatic circulation.

own to have a low biliary clearance. Ter-  
 butaline agent of the β<sub>2</sub>-stimulating type.  
 relative biliary excretion of 0.03-0.04

## Methods

12-H-benflin, 12-H-terbutaline, 12-H-tyridol  
 AB, Södertälje, Sweden  
 analysis of bile  
 unanesthetized rats.  
 1975 95 15

## Editorial Board

F Buchthal  
København

K. Hartala  
Turku

J Jansen  
Oslo

Y Zotterman  
Stockholm

□ S. von Euler  
(Editor) Stockholm

## Editorial office

Acta Physiologica Scandinavica  
Karolinska Institutet  
S-104 01 Stockholm

The "Acta physiologica scandinavica" are published for the Scandinavian Society for Physiology and contain contributions to Physiology, Medical Chemistry or Pharmacology by Scandinavian authors or from Scandinavian laboratories. The articles are published in English, French or German. Each number consists of about 9 printed sheets, 4 numbers forming a volume. Not more than 3 volumes will appear each year. Subscriptions should be mailed to Acta Physiologica Scandinavica, Karolinska Institutet, S-104 01 Stockholm, Sweden.  
Price per volume 88 Sw Cr

Manuscripts from Denmark should be sent to Professor F. Buchthal,  
Universitetets Neurofysiologiske Institut, Juliane Mariesvej 36, DK 2100 København Ø

Manuscripts from Finland should be sent to Professor K. Hartala,  
Fysiologiska Institutionen, Universitetet, Turku.

Manuscripts from Norway should be sent to Dr med Jan Jansen,  
Fysiologisk Institutt, Universitetet Karl Johans Gate 47 Oslo, and

Manuscripts from Sweden should be sent to Professor Y. Zotterman,  
Wenner-Gren Center Konferenssekreterariatet, 23 tr Sveevägen 166, 113 46 Stockholm.

## The Biliary Excretion of $^3\text{H}$ Imulin and $^3\text{H}$ Terbutaline in the Unanesthetized Rat

By

HANS ERIKSSON, WERA HELLSTRÖM and ÅKE RYRFELOD

Received 10 December 1974

### Abstract

ERIKSSON, H., W. HELLSTRÖM and Å. RYRFELOD: The biliary excretion of  $^3\text{H}$ -imulin and  $^3\text{H}$ -terbutaline in the unanesthetized rat. *Acta physiol. scand.* 1975. 95. 1 5

An animal model with permanent cannulation of arterial blood and bile from unanesthetized rats with interrupted enterohepatic circulation is described. Pharmacokinetic data concerning imulin ( $0.2 \mu\text{mol kg}^{-1}$ ) and terbutaline ( $1.8 \mu\text{mol kg}^{-1}$ ) after intravascular injection are presented. The following values for imulin were obtained  $V_d$   $0.23 \text{ ml g}^{-1}$ , plasma clearance  $2.3 \text{ ml min}^{-1} \text{ kg}^{-1}$  and bile clearance  $0.83\text{--}0.64 \text{ ml min}^{-1} \text{ kg}^{-1}$ . Such results are in agreement with data from the literature. For terbutaline the corresponding values were  $0.58 \text{ ml g}^{-1}$ ,  $5.2 \text{ ml min}^{-1} \text{ kg}^{-1}$  and  $0.12\text{--}0.94 \text{ ml min}^{-1} \text{ kg}^{-1}$  respectively.

In most studies concerning biliary excretion in rats anesthetized bile fistula animals have been used. The anesthesia and trauma of the operation may affect the excretory function (Herman *et al.* 1971). The use of unanesthetized rats, which have been allowed to recover from the operation might therefore be of advantage. Various techniques to obtain unanesthetized bile fistula rats with or without interruption of the enterohepatic cycle have been described (Lambert 1965). From a physiological point of view an animal model without interrupted enterohepatic circulation is to be preferred (Light *et al.* 1959). The knowledge concerning biliary clearance and excretion using unanesthetized rats is scanty. In the present investigation the biliary excretion of imulin and terbutaline have been studied using the unanesthetized bile fistula rat, with intact enterohepatic circulation.

Imulin is a polar high molecular compound known to have a low biliary clearance (Schanzler and Hogben 1961). Terbutaline, an antiasthmatic agent of the  $\beta_2$ -stimulating type, is a highly polar amphoteric compound. The cumulative biliary excretion of this drug has been reported recently (Nilsson *et al.* 1973).

### Materials and Methods

#### Compounds

Tris(2,4-dinitrophenyl) sulphate [1-(3,5-dihydroxyphenyl)-2-(4-tert-butylsulfonyl)-1- $^3\text{H}$ -ethanol sulphate] was synthesized at the Research Laboratories, Astra Läkemedel AB, Södertälje, Sweden. Tris(2,4-dinitrophenyl) sulphate, randomly labelled, was obtained from the Radiochemical Centre, Amersham, England. The molar radioactivity of terbutaline and imulin was 13.6 and 0.3 Ci  $\mu\text{mol}^{-1}$  respectively. The radiochemical purity of  $^3\text{H}$ -terbutaline was found to be higher than 0.98 according to thin layer chromatography analysis (see below). The radiochemical purity of imulin was about 0.98 according to the manufacturer.

## Instructions to authors

Manuscripts (2 copies) should be sent to the National Editor in double spacing on size 21 × 30 cm (A 4) with 4 cm margin. A short title (max. 40 letters) may be suggested. As not exceeding 200 words should be submitted.

In general a succinct style and restriction to the necessary of documentation and effectively aids in reducing publication time.

References should be given with full title and name of journals, abbreviated in accordance *Ed. of World List of Scientific Periodicals* with volume number and first and last page numbers.

Figures should not be larger than manuscript pages and sent in as glossy prints in a size that required for reproduction. Lettering should be large enough to permit suitable reproduction preferably of uniform size. When possible diagrams and photomicrographs should extend horizontally rather than vertically in order to save space. Photomicrographs should be calibrated in (not as enlargement factor in figure text). Figure texts should be assembled on separate sheets.

Tables should be kept at minimum both in number and size with text above the table (separate sheets). Single numbers in a series should be replaced by mean and S.D. or mean in the latter case with number of observations.

Key words (5–10) are recommended in order to facilitate indexing.

For abbreviations, units and symbols see special list in the Journal and recent articles.

More detailed instructions to authors are under preparation pending the recommendations Scandinavian Publications Committee.

## The international system of units (SI)

The following symbols and units recommended by the SI are being used in Acta Ph. Scandinavica. Certain units not included in SI will still be permitted.

### SI units with recommended symbols

Units	Symbols
kilogramme	kg
second	s
mole	mol
micro-	μ
milli-	m
nanomole	nmol
picomole	pmol
meter	m
millimeter	mm
micrometer	μm
nanometer	nm
candela	cd
steradian	sr
hertz (frequency)	Hz (s <sup>-1</sup> )
newton (force)	N (kg m/s <sup>2</sup> )
pascal (pressure)	Pa (N/m <sup>2</sup> )
joule (energy)	J (N m)
watt (effect)	W (J/s)
lumen (light flow)	lm (cd sr)
lux (illumination)	lx (lm/m <sup>2</sup> )

### Permitted non-SI units

Units	Symbols
gramme	g
minute	min
hour	h
molarity (mol/liter)	M
(calorie)	cal (4.184 J)
(kilopond)	kp (9.81 N)
(millimeters of mercury)	mm Hg (1.333 bar)
(millibar)	mbar (100 hPa)
curie	Ci
liter	l
milliliter	ml
micro-	μ
degree Celsius	°C

Conversion factors to be given in Methods.

TABLE II. Some pharmacokinetic parameters for insulin ( $0.2 \mu\text{mol kg}^{-1}$ ) and terbutaline ( $1.2 \mu\text{mol kg}^{-1}$ ) administered intravenously to unanesthetized male bile fistula rats. A two compartment open model has been used to characterize the pharmacokinetic profiles of the compounds.

parameter	Insulin	Terbutaline
( $\mu\text{mol ml}^{-1}$ )	2.62	34.5
( $\mu\text{mol ml}^{-1}$ )	0.18	0.27
( $\text{h}^{-1}$ )	7.22	6.42
( $\text{h}^{-1}$ )	0.54	0.56
( $\text{h}^{-1}$ )	0.23	0.58
$k_2$ ( $\text{ml g}^{-1}$ )	2.3	5.2
renal clearance ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	0.03-0.04	0.13-0.96
hepatic clearance (hepatic) ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	0.1	1.2
cytochrome P-450 ratio		

1971) anaesthesia and trauma of operation may affect the excretory function. Further experiments are therefore needed in order to clarify differences in excretory function between anesthetized and unanesthetized animals.

Technical assistance provided by Mrs Malin Johansson is gratefully acknowledged.

### References

- KIMURA, N. O., E. HANSEN, C. H. RAMBA and Å. RYSELDT The tissue distribution of  $^{125}\text{I}$ -terbutaline (Bricton<sup>®</sup>) in mice. *Acta physiol. scand.* 1972. 84 40-47.
- JOSE, D. L., C. A. LAWLER, L. B. CALVIN and E. M. GREEN, Mechanisms of bile secretion. *Amer J Physiol* 1952. 171 62-74.
- JOSE, D. L. and H. HELLER, The mechanism of water diuresis in normal rats and rabbits as analyzed by insulin and diuretic diuretics. *J Physiol (Lond)* 1945. 103. 469-480.
- FEDERMAN, M., The creatinine, urea and hippuric clearance in the rat. *Amer J Physiol* 1947. 148. 357-391.
- FEDERMAN, M. and C. A. LYNNSTON, The estimation of renal function in the rat by the use of diuretic and insulin. *Amer J Physiol* 1942. 137 564-569.
- JOSE, D. L., R. MAGARAS and G. LEVY Relationship between drug concentration in plasma or serum and the amount of drug in the body. *J pharm. Sci.* 1969. 58 193-197.
- HEDNER, A. H., R. REEDER, D. SMALL and R. EDWARDS, The effects of surgery on bile secretion and composition. *Surg Forum* 1971. 22 378-380.
- LAMBERT, R., Surgery of the digestive system in the rat. Charles Thomas Publisher, Springfield, Illinois, U.S.A. 1965.
- LIGHT, H. O., C. WYMAN and H. M. VASE, Interruption of the splanchnic circulation and its effect on rat bile. *Amer J Physiol* 1959. 197 1330-1332.
- MELBY, H. T., C. O. A. PERSSON, K. PERSSON, K. TSONDE and Å. RYSELDT The metabolism of terbutaline in dog and rat. *Neuropharmacology* 1973. 12 615-623.
- ROTHMAN, S., J. C. K. LOO and M. ROWLAND, Shortcomings in pharmacokinetic analysis by considering the body to exhibit properties of single compartment. *J pharm. Sci.* 1964. 53 117-123.
- ROWLAND, M., Application of clearance concepts to some literature data on drug metabolism in the isolated perfused liver preparation and in vivo. *Europ. J Pharmacol* 1972. 17 352-356.
- SCHMIDT, L. S. Secretion of organic compounds. In: *Handbook of physiology: Alimentary canal* 1964. Sect. 6 Vol. 5, Chap. 4. pp. 2433-2449.
- SCHMIDT, L. S. and C. A. HOOVER, Biliary excretion of insulin, urea and mannitol, analysis of bile formation. *Amer J Physiol* 1961. 200. 1087-1090.
- WELLS, J. R. and J. A. JONES, Routine direct measurement of arterial pressure in unanesthetized rats. *Proc. Soc. exp. Biol (N.Y.)* 1960. 104 646-648.
- ZIL, A., Thyroid function in relation to bile lipids and bile acids. *J Afr. med. J* 1959. 11. 618.

TABLE I Bile flow ( $\text{ml min}^{-1} \text{kg}^{-1}$ ) and biliary clearance ( $\text{ml min}^{-1} \text{kg}^{-1}$ ) of total radioactivity and unchanged drug after intraarterial injection of  $^3\text{H}$  inulin ( $0.2 \mu\text{mol kg}^{-1}$ ) or  $^3\text{H}$  terbutaline ( $1.8 \mu\text{mol kg}^{-1}$ ) in unanesthetized male bile fistula rats. The values given are means  $\pm$  S.D. ( $N=4-5$ ).

Time (min)	Control Bile flow	Inulin		Terbutaline		
		Bile flow	Clearance	Bile flow	Clearance (total)	Clearance (unchanged)
-60-0	$0.11 \pm 0.01$	$0.11 \pm 0.03$	—	$0.12 \pm 0.03$	—	—
0-20	$0.12 \pm 0.02$	$0.12 \pm 0.04$	—	$0.13 \pm 0.03$	—	—
20-40	$0.11 \pm 0.03$	$0.10 \pm 0.02$	0.03	$0.14 \pm 0.06$	2.51	0.17
40-60	$0.13 \pm 0.02$	$0.10 \pm 0.02$	0.04	$0.13 \pm 0.05$	4.24	0.98
60-80	$0.11 \pm 0.02$	$0.12 \pm 0.03$	0.04	$0.13 \pm 0.04$	4.24	0.73
80-100	$0.11 \pm 0.03$	$0.10 \pm 0.03$	0.04	$0.14 \pm 0.05$	1.67	0.69
100-120	$0.09 \pm 0.02$	$0.07 \pm 0.03$	0.03	$0.11 \pm 0.03$	0.79	0.29
120-140	$0.07 \pm 0.01$	$0.10 \pm 0.01$	0.03	$0.10 \pm 0.03$	0.42	0.13
140-160	$0.08 \pm 0.01$	$0.09 \pm 0.01$	0.03	$0.09 \pm 0.01$	1.34	0.16
160-180	$0.07 \pm 0.01$	$0.08 \pm 0.02$	0.03	$0.07 \pm 0.02$	0.38	0.1

The control rats were treated exactly as the dosed rats as regard to bile and blood sampling.

urine collecting periods) and Friedman (1947) ( $1.1-4.7 \text{ ml min}^{-1} \text{kg}^{-1}$  obtained in rats anesthetized partly during urine collecting periods for blood sampling).

The distribution volume ( $V_d$ ) of inulin was  $0.25 \text{ ml g}^{-1}$  which is about the same as the relative extracellular water content of the body. The uptake into blood cells was low (Table II) which is a well known fact.

With terbutaline a dose of  $1.8 \mu\text{mol kg}^{-1}$  ( $0.5 \text{ mg kg}^{-1}$ ) was used which is a dose of pronounced pharmacodynamic activity. The bile to plasma concentration ratio of unchanged terbutaline varied from 1.2 to 7.5 and the biliary clearance from 0.12 to  $0.98 \text{ ml min}^{-1} \text{kg}^{-1}$  (2 to 5 times the bile flow), indicating a concentrative transfer of terbutaline from plasma to bile. At physiological pH values, terbutaline is in the cationic form and it is known that several cations are actively secreted into the bile (Schanker 1968). The biliary clearance of total radioactivity ranged from 0.38 to  $4.36 \text{ ml min}^{-1} \text{kg}^{-1}$ . This indicates that the formed metabolite(s) is excreted more extensively than the unchanged drug. The cumulative excretion of radioactivity was larger than 0.3 of the administered dose of radioactivity. This is in agreement with values obtained by Nilsson *et al.* (1973) in experiments with unanesthetized rats with interrupted enterohepatic circulation.

The plasma clearance of unchanged terbutaline was  $5.2 \text{ ml min}^{-1} \text{kg}^{-1}$  which reflects excretion (biliary and urinary) as well as biotransformation of terbutaline.

The erythrocyte to plasma concentration ratio for terbutaline was about one. This indicates that the substance can either penetrate the membrane of red blood cells or attach to their surfaces. The distribution volume ( $V_d$ ) of terbutaline was  $0.58 \text{ ml g}^{-1}$  which is about the same as the relative water content of the body. Autoradiographic studies have shown that terbutaline is not concentrated in any specific tissue or organ with the exception of liver and kidney (Bodin *et al.* 1972).

Results obtained in the present investigation agree mainly with those obtained by other investigators in experiments with unanesthetized rats. As has been shown by e.g.

TABLE II. Some pharmacokinetic parameters for iothol (0.2  $\mu\text{mol kg}^{-1}$ ) and terbuthalins (1.8  $\mu\text{mol kg}^{-1}$ ) administered intravenously to unanesthetized male bile fistula rats. A two compartment open model has been used to characterize the pharmacokinetic profiles of the compounds.

Parameter	Iothol	Terbuthalins
A ( $\mu\text{mol ml}^{-1}$ )	8.62	34.5
B ( $\mu\text{mol ml}^{-1}$ )	0.18	0.27
$\alpha$ ( $\text{hr}^{-1}$ )	7.22	6.42
$\beta$ ( $\text{hr}^{-1}$ )	0.34	0.56
$V_d/\beta$ ( $\text{ml g}^{-1}$ )	0.25	0.38
Plasma clearance ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	2.3	5.2
Bile clearance (mechanical) ( $\text{ml min}^{-1} \text{kg}^{-1}$ )	0.03-0.04	0.12-0.30
Erythrocyte/plasma ratio	0.1	1.2

1971) anesthesia and trauma of operation may affect the excretory function. Further experiments are therefore needed in order to clarify differences in excretory function between anesthetized and unanesthetized animals.

The technical assistance provided by Mrs Marian Johansson is gratefully acknowledged.

### References

- BOON, N. O., E. HAMMON, C. H. RAMBA and Å. RYSELEDT The tissue distribution of  $^3\text{H}$ -terbuthalins (Brucap®) in mice. *Acta physiol. scand.* 1972, **34**, 40-47.
- COOK, D. L., C. A. LAWLER, L. D. CALVIN and D. M. GREEN, Mechanism of bile formation. *Amer. J. Physiol.* 1952, **171**, 62-74.
- DICKER, S. H. and H. HELLER, The mechanism of water diuresis in normal rats and rabbits as analyzed by iothol and diiodine diacetates. *J. Physiol. (Lond.)* 1943, **103**, 449-460.
- FRIEDMAN, M. The cruciata, iothol and iopodate clearance in the rat. *Amer. J. Physiol.* 1947, **143**, 357-391.
- FRIEDMAN, R. M. and C. A. LIVINGSTONE, The extension of renal function in the rat by the use of diacetol and iothol. *Amer. J. Physiol.* 1943, **137**, 564-569.
- GRALER, M. R. NAGASAWA and G. LEVY Relationship between drug concentration in plasma or serum and the kinetics of drug in the body. *J. Pharm. Sci.* 1969, **58**, 193-197.
- HOLMAN, A. H. R. KERNICK, D. SMALL and R. SCHAM, The effects of surgery on bile secretion and composition. *Surg. Forum* 1971, **22**, 378-380.
- LAURENT, R., *Surgery of the digestive system in the rat*. Charles Thomas Publisher Springfield, Illinois, U.S.A. 1965.
- LOMT, H. G., C. WITMER and H. M. VALL, Interruption of the enterohepatic circulation and its effect on the bile. *Amer. J. Physiol.* 1959, **197**, 1310-1332.
- NELSON, H. T. C. O. A. PERSSON, K. PERSSON, K. THORST and Å. RYSELEDT The metabolism of terbuthalins in dog and rat. *Konvens* 1973, **2**, 615-623.
- REICHLIN, S. J. C. X. LOO and M. ROWLAND, Shortcomings in pharmacokinetic analysis by considering the body to exhibit properties of a single compartment. *J. Pharm. Sci.* 1968, **59**, 117-123.
- ROWLAND, M., Application of clearance concepts to some literature data on drug metabolism in the isolated perfused liver preparation and in vivo. *Europ. J. Pharmacol.* 1972, **17**, 352-356.
- SCHAMER, L. S. Secretion of organic compounds. In: *Handbook of physiology: Abnormal canal* 1968, Sect. 6, Vol. 3, Chap. 4, pp. 3433-3449.
- SCHAMER, L. S. and C. A. HOOD, Biliary excretion of urea, sucrose and mannitol: analysis of bile formation. *Amer. J. Physiol.* 1961, **200**, 1087-1090.
- WILKIN, J. R. and J. A. JONES, Routine direct measurement of arterial pressure in unanesthetized rats. *Proc. Soc. exp. Biol. (N.Y.)* 1962, **104**, 646-648.
- van ZYL, A. Thyroid function in relation to bile lipids and bile acids. *S. Afr. med. J.* 1979, **55**, 612.



## The Permeability of Fish Gills with Comments on the Osmotic Behaviour of Cellular Membranes

By

JOHAN B. STEEN<sup>1</sup> and SVERRE STRAY PEDERSEN

Received 9 January 1975

---

### Abstract

---

STEEN, J. B. and STRAY PEDERSEN. *The permeability of fish gills with comments on the osmotic behaviour of cellular membranes* Acta physiol. scand. 1975 95 6-20

The permeability of eel gills to ions, lipid-soluble and -insoluble substances has been measured under steady-state conditions. The results obtained support the multi-pathway model for transcapillary transport (Stray-Pedersen and Steen 1975). The gills are 25 times more permeable to THO than to  $\text{Na}^+$  or  $\text{K}^+$ . Metabolic inhibitors do not affect the transfer of substances across the gills. Exposure to EDTA or MS-22 changes the permeability properties to resemble those of an aqueous barrier. The gills are 10 times less water permeable than the capillaries of the *rete mirabile*.

---

Present concepts on the mechanisms of capillary wall penetration are largely based on informations obtained in experiments on perfused organs, particularly on mammalian skeletal muscles. The permeability constants in these experiments have been calculated on the basis of studies of osmotic transients or from different types of clearance studies during transient conditions (Crone 1970).

We wanted to test some of these concepts on simpler systems in which the phenomena of vessel wall penetration could be studied more directly and during steady-state conditions. Such studies have been performed in the *rete mirabile* of the eel (Stray-Pedersen and Steen 1975, Stray-Pedersen 1975). These investigations showed that the mechanisms of transcapillary diffusion are more complex than generally assumed. As an alternative to the single-pore theory (Pappenheimer *et al* 1951, Pappenheimer 1953) a multipathway theory for diffusion across capillary membranes was suggested (Stray-Pedersen and Steen 1975).

The present paper reports experiments carried out on the gills of the eel. Fish gills are specialized for gas exchange between blood and water. The internal and external media are exposed to each other across a large, thin membrane made up by the walls of the gill lamellae. These are thin, flat, hollow sheets which are perfused by blood and irrigated by

---

<sup>1</sup> Present address: Physiological Section, Institut of Medical Biology University of Tromsø, 9000 Tromsø, Norway

water in the eel the lamellar wall is 2 to 5  $\mu\text{m}$  thick and consists of a layer of flattened endothelial cells and two or more layers of epithelial cells. Between the endothelium and the epithelium various amounts of interstitial tissue are imposed. The intercellular clefts between the endothelial cells have tight junctions (Hughes and Grimstone 1965) similar to the capillaries of the brain of mammals (Brightman and Reese 1969). Direct determinations of the ionic composition of the gill tissue have not been performed. However we have measured the concentrations of  $\text{Na}^+$ ,  $\text{K}^+$  and  $\text{Cl}^-$  in squeezed gill filaments disintegrated by sulphuric acid (Stray-Pedersen and Nicolaysen, 1975) and found values of about 20, 60 and 80 meq/l respectively.

We are aware of the fact that the structure and function of the gill membrane are more complex than the capillary membrane of the *rete mirabile*. The aim of this investigation, however, was not primarily to describe the function of the normal gill as such, but rather to use the gill as a convenient preparation for permeability studies and thereby obtain information about the permeability properties of its capillary membranes. In this preparation, as was also the case in the *rete*-preparation, the permeability of a substance could be determined directly from measurements of its concentrations on both sides of the capillary membrane during steady-state conditions. Since the maintenance of normal capillary permeability characteristics have been shown to be dependant on  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  (Chambers and Zweifel 1940, Wilbrandt *et al.* 1956, Nicolaysen 1971 a, b, Stray-Pedersen 1975), the effects due to removal of these ions were studied. The cells of the gill membrane are known to be metabolically very active, the organ playing an important role in for instance the osmoregulation (Maretz 1971, Motals and Garcia-Romeu 1972). It was therefore of great interest to study the effects of metabolic inhibitors, temperature and the drug MS-222 which was used as an anaesthetic in the present experiments. The results obtained were compared to those obtained in the *rete*.

## Methods

### Animals

The eels are exclusively silver eels adapted to fresh water weighing between 100–200 g.

### Surgery

The eel is made unconscious by blow on the head and fixed in a holder (Særen 1963). A longitudinal opening exposed the ventral aorta which was cannulated for perfusion and tied off just anterior to the first branches off to the two posterior gill arches. The eel is turned with its dorsal side up and the spinal cord was removed to expose the dorsal aorta. This was cannulated and ligated so that only the two posterior arches were available for perfusion. Blood vessels to the brain and facial region arose from the two anterior gill arches only and no branching occur from the part of the gill circulation which was perfused in these experiments.

### Perfusion

The gills are ventilated with water through tubes inserted into the mouth of the eel. The water is delivered either directly from the tap or by recirculation by an air plastic pump. The rate of ventilation had to be above 100 ml/min to ensure adequate irrigation of all parts of the gills.

### Perfusion

The gills were perfused with heparinized horse plasma prepared from fresh horse blood containing 20 I.U. heparin per ml. The plasma was stored at 20°C and used within 90 days.

TABLE 1 The ionic composition and osmolarity of horse plasma and eel plasma.

	K <sup>+</sup> mM/l	Na <sup>+</sup> mM/l	Cl <sup>-</sup> mM/l	Ca <sup>++</sup> mM/l	Mg <sup>++</sup> mM/l	Osmola (mOsm)
Horse plasma	3.3-3.6	125-135	95-102	2.2-2.5	1.1-1.2	270-290
Eel plasma	3.1-3.5	149-167	75-105	2.5-2.7	1.1-1.1	280-310

During perfusion with horse plasma the preparation was apparently very well maintained for a hour. Although the circulatory pathway had no leakages, lymphatic leakage did bother us initially. tion of 1 mg adrenalin per 100 ml perfusate efficiently prevented this. It also caused the perfusate to circulate the lamellae and not the lymphatic shunts of the gills. (Steen and Krøyer 1965).

The composition of horse plasma has been compared in that of eel plasma in Table 1. The preparation was perfused at 0.1 ml/min and at very low pressure (less than 5 cm H<sub>2</sub>O). The outflow was collected in known periods of time and weighed. Under normal conditions inflow and outflow volumes were within 2% of each other.

The normal cardiac output, and therefore also the total gill blood flow in eels of this size, is 1 to 2 ml/min. When our preparation was perfused at these rates, the exchange of many substances was too slow to be detected with accuracy. A perfusion rate of 0.1 ml/min was found to be optimal for our purpose. At this rate the exchange loss of substances across the gills was never less than one half of that observed at 1 ml/min. This shows that such a reduction of flow reduced the exchange area by at least 50%.

At the selected rate of perfusion and dead-space volume of the tubes, steady state conditions were established within 30 min after the onset of perfusion. It could be shown experimentally that the contents of the preparation were saturated with the isotopes (except for <sup>42</sup>K) in the course of this period.

#### *Test substances*

As test-substances were used urea <sup>14</sup>C, sucrose <sup>14</sup>C, dextran-75 000- <sup>14</sup>C (NEC 218B, MW 60 000), ethanol- <sup>14</sup>C, antipyrine- <sup>14</sup>C, tritiated water (THO), <sup>42</sup>K, <sup>44</sup>Na+ <sup>22</sup>Na and Cl<sup>-</sup>. The substances under study were usually added to the perfusate, but in some experiments they were added to the venous blood.

Some relevant physico-chemical data of the test-substances are listed in Table II.

#### *Analytical methods*

Na<sup>+</sup> and K<sup>+</sup> was measured by standard flame photometry (Eppendorf). At concentrations within the range of normal plasma concentrations the measurements were accurate (within  $\pm 2\%$ ).

Cl<sup>-</sup> was determined with an accuracy of  $\pm 1\%$  on chloridometer (electrical titration) (Eel).

The isotopes were counted in a Packard liquid scintillation counter. Precautions were taken to avoid cross-counting. The input activities chosen were high enough to give relatively small counting errors. Additional inaccuracies by dilution gave values which were accurate within  $\pm 3\%$ .

The osmolarity of samples was measured with an accuracy of  $\pm 2$  mOsm with an osmometer (Ad Digimatic mod 3 D).

#### *The measurement of exchange*

The exchange of a substance across the gill barrier was estimated from its concentrations in the perfusate (the output and input sides). This method is valid only if

- 1) The substance does not accumulate in the gill tissue.
- 2) The perfusate is not diluted due to osmosis or to admixture with lymph during perfusion.

Ad 1) The possible occurrence of tissue accumulation of test-substances was investigated by comparing the amount of substance appearing in the ventricle with that disappearing from the perfusate. No accumulation could be detected for any of the substances tested except for <sup>42</sup>K.

Ad 2) Dilution and lymph admixture was checked by comparing the concentration of dextran 75 to which the gill membrane was shown to be impermeable, in the perfusate at the input and output sides. Since no difference was observed, no dilution could have taken place.

THO was used as test-molecule in all experiments and consequently served as a reference. The exchange of any other substance was related to the THO-exchange measured simultaneously.

TABLE II. Relevant physico-chemical data of the test-substances<sup>a</sup>

Substance	MW	Molecular (ionic) radius (Å)	Olve oil/water partition coefficient	D <sub>20</sub> °C 10 <sup>-6</sup> cm <sup>2</sup> sec <sup>-1</sup>	D <sub>9</sub> /D <sub>20</sub> cm <sup>2</sup> sec <sup>-1</sup>
THO	20	1.3	0.0007	2.44	1
NaCl (Na <sup>+</sup> )	24	—	Insoluble	1.48	0.61
Cl <sup>-</sup>	35	—	Insoluble	1.92	0.79
Na <sup>+</sup>	42	—	Insoluble	1.92	0.79
Ethanol	46	2.4	0.022	1.34	0.51
Urea	60	2.6	0.00015	1.38	0.57
Antipyrin	188	4.1	0.012	0.68	0.28
Sucrose	342	5.6	Insoluble	0.52	0.21
Dextran-75 000	60 000-80 000	60	Insoluble	0.036	0.013

<sup>a</sup>For details see Kirk-Pedersen and Steen 1973.

### Equations

The permeability ( $P$ ) and diffusion ( $D$ ) constants were calculated on the basis of the values obtained from the loss of substance during the passage through the gills. Since the ventilation flow (water flow) is more than 1000 times as great as the perfusion flow the concentrations of test-substances in the static were assumed to be constant. For the time  $t$  in which there is a net diffusion from the perfusate water of test-substance, leaving concentration in water of zero and the concentrations  $C_1$  and  $C_2$  in input and output plasma solution, we get

$$C_2 = C_1 e^{-kt} \quad (1)$$

$$k = \frac{\ln(C_1/C_2)}{t} \quad (2)$$

we assume that the concentration falls exponentially during the time  $t$  it takes the perfusate to pass through the gills. The mean concentration of the substance in the perfusate (= mean concentration difference across the gill membrane),  $\bar{\Delta C}$ , may be found by integration of Eq. (1).

$$\bar{\Delta C} \cdot t = \int_{C_2}^{C_1} C \cdot \ln C \, dC \quad (3)$$

$$\bar{\Delta C} \cdot t = C_2 - C_1 e^{-kt} \quad (4)$$

substituting from Eqs. 1 and 2 into Eq. 4 gives:

$$\bar{\Delta C} = \frac{C_1 - C_2}{\ln(C_1/C_2)} \quad (5)$$

according to Fick's law of diffusion:

$$\dot{Q} = D \frac{\bar{\Delta C} A}{l} \quad (6)$$

the amount of substance transported per unit time,  $A$  the area available to exchange,  $l$  the thickness of the gill barrier,  $D$  the diffusion coefficient.

$\dot{Q}$  also equal to:

$$\dot{Q} = (C_1 - C_2) w \quad (7)$$

with  $w$  the flow of perfusate.

The diffusion constant or the permeability constant is obtained by combining Eqs. 5, 6 and 7

$$D = \frac{wl}{A} \ln \frac{C_1}{C_2} \quad (8)$$

or

$$P = \frac{W}{A} \ln \frac{C_1}{C_2}$$

The units are  $W = \text{cm}^3 \text{ sec}^{-1}$ ,  $l = \text{cm}$  and  $A = \text{cm}^2$  whereas any units can be used for  $C_1$  and  $C_2$  as long as the same units are used in nominator and denominator.  $P$  has the units of  $\text{cm}^2 \text{ sec}^{-1}$  and  $P$  of  $\text{cm} \text{ sec}^{-1}$ . The result corresponds to the equation derived by Crone (1963).

The values for the structural parameters  $l$  and  $A$  are difficult to estimate. The area of the lamellae of the two posterior gill arches was measured on eels of the same weight range as the eels used in the experiments by the methods of Hughes (1966). However, we found no good way to assess the properties of lamellae actually perfused in each experiment. By using the anatomical values for  $A$  and  $l$ , 200  $\text{cm}^2$  and  $5 \cdot 10^{-4} \text{ cm}$  respectively  $D$  and  $P$  are therefore most likely underestimated. In most cases we expressed the permeability by the factor  $\ln(C_1/C_2)$ , which we termed the permeability factor.

#### *Accuracy of permeability values*

The accuracy of  $\ln(C_1/C_2)$  depends on the difference between  $C_1$  and  $C_2$ . This means that the accuracy will be higher the higher the permeability. Generally the difference in concentration between input and output must exceed 3% of the input value to yield a permeability value of reasonable accuracy. Since the exchange of the ions and the lipid-insoluble test-molecules was of this magnitude, several samples were required in each experiment to obtain reliable values.

## Results

### *Perfusions with normal plasma*

In more than 100 expts. the gill preparation was perfused with horse plasma containing THO and one or more of the test-substances. Each experiment lasted for 2 to 7 h. The results obtained in a typical experiment are shown in Fig. 1. As seen from the figure there was a tendency for the loss of substances from the perfusate to be reduced with time. This tendency was most pronounced for  $^{42}\text{K}$ . Except for this ion, the mutual relationship between the exchange of the different substances was maintained throughout an experiment. No differences between the osmolarity of the input and the output perfusates were obtained in these experiments.

The comparatively great loss of  $^{42}\text{K}$  from the perfusate, however, was resulted in an accumulation of this isotope in the gill tissue rather than to flux across the gill barrier. This fact became evident when the loss of  $^{42}\text{K}$  from the perfusate was compared to the gain in the ventilate. Since very high activities of the isotope were required, only 4 expts. of this type were performed. It turned out that about 10% of the amount of isotope lost from the plasma was recovered in the ventilate. We also counted the  $^{42}\text{K}$ -activity in weighed pieces of gill tissue after about 3 h of perfusion with the  $^{42}\text{K}$ -activity of the perfusate. The specific activity of the tissue was found to be about 12 times as great as that of the perfusate. The total tissue activity agreed to within 15% to the total amount of  $^{42}\text{K}$  lost during the preceding perfusion.

TABLE III The range of the loss of test-substances from the perfusate (expressed in per cent of input concentration) obtained in about 100 expts. (20–22°C, flow 0.1 ml/min, perfusate: modified horse plasma. The concentration of test-substance in the ventilate was zero.)

Substance	THO	Antipyrine	Ethanol	Urea	Sucrose	$^{42}\text{K}$	Na	Cl	D-75 000
disappeared	35–80	10–40	30–75	10–15	0	10–30	2–4	2–4	0

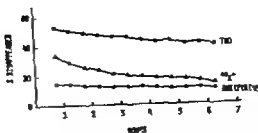


Fig. 1. Results from an experiment showing the typical change in the extraction of antineoplastic substances (expressed in per cent of the corresponding initial concentration) during continuous perfusion with renal plasma. Note the pronounced reduction in the loss of  $^{42}\text{K}$  compared to that of THO and acetate during the perfusion. In this experiment the gills were irrigated with tap water ( $t = 7^\circ\text{C}$ ).

In Table III the percent disappearance from the perfusate of the different test-substances (except for  $^{42}\text{K}$ ) is quoted. The values represent the ranges obtained in about 100 expts. The differences in the exchange obtained in the experiments was most probably due to differences in the exchange area available, since the mutual relationship between the exchange of different substances showed far less variation. Therefore, the diffusion constants of the gill membrane quoted in Table IV were based on data from the experiments showing the highest exchange, *i.e.* where the exchange area was closest to the anatomical area. In the same table the relative diffusion anatomical area. In the same table the relative diffusion coefficients,  $D_g/D_{\text{THO}}$ , are given and compared to those obtained in the *rete*. Comparison with Table II shows that the permeability properties are very different from those of an aqueous barrier.

The exchange of the substances across the gill membrane in the direction from ventilate to perfusate was also investigated. To this end the ventilate containing the test-substances were recirculated and the concentrations recorded in the perfusate outflow. The exchange of THO, urea and  $^{22}\text{Na}$  was the same as in the perfusate-to-ventilate direction, whereas the exchange of  $^{42}\text{K}$  was found to be 10 times smaller.

TABLE IV. Diffusion constants and diffusion rates of the gill membrane compared to those of the *rete* membrane.

	Gills Diffusion constants ( $20^\circ\text{C}$ ) $D_g \cdot 10^6 \text{ cm}^2 \text{ sec}^{-1}$	Gills $D_g/D_{\text{THO}}$	<i>Rete</i> <sup>a</sup> $D_R \cdot 10^6 \text{ cm}^2 \text{ sec}^{-1}$	<i>Rete</i> $D_R/D_{\text{THO}}$
THO	7.3	1	66.4	1
$^{42}\text{K}$	—	—	1.84	0.027
$^{22}\text{Na}$ ( $\text{Na}^+$ )	0.3	0.04	1.44	0.022
$\text{Cl}^-$	0.3	<0.04	—	—
Urea	1.3	0.15	1.74	0.026
Sucrose	—	—	0.58	0.009
Ethanol	7.3	1.0	68.0	1.01
Acetypyrene	3.6	0.5	57.2	0.86

<sup>a</sup>Assumes thickness of the *rete* capillary barrier of  $2 \mu$ . Data from Stray-Pedersen and Steen (1975).

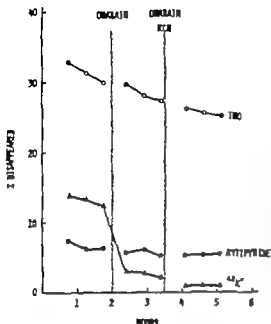


Fig. 2. The effect of ouabain and KCN. The extraction of THO and antipyrine (expressed as percent of the corresponding input concentration) did not change significantly during the experiment compared to that observed during normal conditions (see Fig. 1). However a marked reduction in the loss of  $^{42}\text{K}^+$  from the perfusate was observed in plasma containing 2 mM/l of ouabain was introduced. The loss of  $^{42}\text{K}$  appeared to be further reduced when 5 mM/l of KCN was added to the perfusate. In this experiment the gills are irrigated with tap water ( $t=7^\circ\text{C}$ ).

### Effect of metabolic inhibitors

The effects of the metabolic inhibitors, KCN and ouabain (strophantidine), on the exchange across the gill membrane were studied in 6 experiments. After two hours of normal perfusion we switched to a perfusate containing 5 mM/l of KCN and/or 2 mM/l of ouabain. The results from one typical experiment are shown in Fig. 2.

As may be seen in the figure the introduction of ouabain (and KCN) lead to a marked reduction in the disappearance of  $\text{K}^+$  whereas the disappearance of the other test substances continued unchanged. When the exposure to the inhibitors had lasted for about 30 min the disappearance of  $\text{K}^+$  corresponded to that of  $^{22}\text{Na}^+$  and  $\text{Cl}^-$ .

Addition of KCN and/or ouabain to the ventilate did not lead to any changes in the exchange across the gill barrier of  $\text{K}^+$  or of any other substance under study.

### Effect of temperature

In 18 expts. the temperature of the ventilate was changed from  $5^\circ\text{C}$  to  $20^\circ\text{C}$  every other hour during a perfusion. At each temperature the permeability factor  $\ln(C_i/C_o)$ , was calculated for the test-substances.

The effect of temperature was expressed as the change of the permeability factor caused by a  $10^\circ\text{C}$  change in temperature. This permeability change was called the  $Q_{10}$ . In Table V the  $Q_{10}$ -values obtained for the different test-substances are quoted and compared to the  $Q_{10}$ -values of the corresponding free diffusion coefficients in water.

It is clear that  $Q_{10}$  for ions are similar to the  $Q_{10}$  of free diffusion while the  $Q_{10}$  for water and particularly for more lipid soluble substances is higher.

Experiments at varying temperature in the presence of EDTA will be described in the next section.

TABLE V  $Q_{10}$ -values for the permeabilities of the test-substances obtained during perfusions with normal plasma and with plasma containing EDTA compared to the  $Q_{10}$ -values for the corresponding free diffusion coefficients.

	THO	Antipyrine	Ethanol	$^{22}\text{Na}$	$^{42}\text{K}$	Cl	Urea
Normal plasma	2.1	3.1	2.1	1.4	1.5	—	—
Plasma 4 mM/l EDTA	1.4	1.7	1.3	1.3	1.5	1.2	1.1
Temperature coefficients for the free diffusion coefficients	1.35	1.35	1.35	1.35	1.35	1.35	1.35

#### The effect of removal of $\text{Ca}^{++}$ and $\text{Mg}^{++}$

Calcium and magnesium ions are known to be of importance for maintenance of normal permeability and cell adhesivity. The effects due to removal of these ions were tested in experiments by adding the chelating agent, EDTA (as  $\text{Na}_2\text{-EDTA}$ ), to the perfusate. EDTA was added in concentrations which corresponded to 120% of the total content of Ca and Mg in plasma. The results obtained in a typical experiment are shown in Fig. 3. EDTA did not affect the exchange of THO ethanol or antipyrine. The exchange of  $^{42}\text{K}$  was (apparently) reduced, whereas the exchange of  $^{22}\text{Na}$  Cl urea and sucrose was dramatically increased. After 2 h of an EDTA-perfusion the ratios between the permeabilities of the test-substances had become strikingly similar to those between the free diffusion coefficients (Table VI).

Attempts to reverse the EDTA-effects were performed in many of these experiments. It turned out that if normal plasma perfusates were reintroduced after an EDTA-exposure of 15 min or less, normal permeability properties could be restored.

As described earlier the activity of  $^{42}\text{K}$  in the gill tissue was 12 times higher than in the perfusate after 3 h of perfusion. When these experiments were continued for one hour with a perfusate containing sufficient EDTA to chelate all  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  the tissue activity of  $^{42}\text{K}$  was found to be 7 times higher than the perfusate activity. When after 3 hours of perfusion with normal plasma a perfusate containing the same amounts of EDTA but with 2 mM/l ouabain in addition, the tissue activity was found to be only 2 times higher than

TABLE VI  $D_p/D_{\text{free}}$ -ratios obtained in gills treated with EDTA and MS-222 compared to the  $D_p/D_{\text{free}}$ -ratios of normal gills and of water (25°C).

	Gills			Water (i.e. free diffusion)
	EDTA	MS-222	Normal plasma	
THO	1.0	1.0	1.0	1.0
Na	0.55	0.43	0.04	0.61
Cl	0.67	0.53	0.04	0.79
Urea	0.63	0.5	0.15	0.57
Sucrose	0.33	—	—	0.21
Ethanol	—	—	1.0	0.51
Antipyrine	0.5	—	0.5	0.25



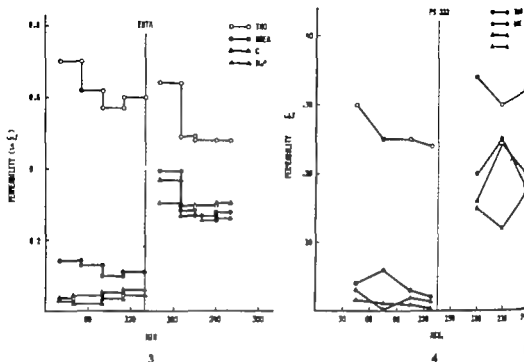


Fig. 3 The effect of EDTA. When EDTA was added to the perfusate in a concentration of about 1% of the total content of Ca and Mg in plasma, the permeability of urea, Cl<sup>-</sup> and Na<sup>+</sup> increased markedly. The THO-permeability however appeared to remain fairly unchanged. In this experiment the gills were irrigated with water at 20°C.

Fig. 4 The effect of MS222. The addition of 2 g/l of MS222 to the ventilate lead to a marked increase in the permeability of urea, Cl<sup>-</sup> and Na<sup>+</sup> whereas the exchange of THO was not affected. In this experiment the gills were irrigated with water at 20°C.

the perfusate activity. In this latter case release of <sup>42</sup>K from the gill tissue was shown to take place by the fact that the <sup>42</sup>K-activity was temporarily about 50% higher in the perfusate than in the input.

The effect of temperature was also examined in gills treated with EDTA sufficient to give significant changes in permeabilities. As shown in Table V the  $Q_{10}$  for the test-substances (including THO and the lipid-soluble molecules) was very similar to that of the corresponding free diffusion coefficients.

#### *The effect of the local anaesthetic MS-222*

MS-222 is an isomer of benzocaine (methanesulphonate of meta-aminobenzoic acid) and is widely used as an anesthetic for aquatic animals. It is added to water in ratios between 1:1000 and 1:10000.

The effect of MS-222 (Sandoz) on the exchange was studied in 14 experiments. The anaesthetic was added either to the ventilate or to the perfusate in concentrations of 0.1 to 0.2 g/l.

During perfusions with plasma containing MS-222 no changes could be observed in the exchange of the test-substances. When the drug had been added to the ventilate, however, the properties of the gill membrane were rather dramatically affected.

similar to those obtained with EDTA (Fig. 4 and Table VI). In order to show that the effects observed were not due to the low pH in the MS-222 ventilate, the gills were ventilated with water to which HCl had been added to match the pH-reduction caused by MS-222 (about pH 3.5). No effects could be observed in these perfusions (3 expts.). We also neutralized the water containing MS-222 by addition of NaOH (2 expts.) but the anaesthetic maintained its effects.

In some experiments MS-222 caused an initial increase in the THO-permeability (Fig. 4). It appeared that this effect occurred at different doses of MS-222 in different preparations. The effects of MS-222 were reversible when the exposure lasted less than 30 min at 20°C.

### Discussion

The permeability studies performed on the *rete mirabile* (Stray-Pedersen and Sten 1975) indicated the existence of three different pathways available to transport across the capillary membrane: 1. One with lipid character allowing the passage of lipid-soluble molecules, 2. "Water-pores" through which only water (or THO) may pass, and 3. Pores allowing the passage of hydrophilic molecules as well as of water. The latter pores were suggested to be equivalent to the Pappenheimer pores represented by the intercellular junctions, whereas the other two pathways are apparently confined to the endothelial cells proper. A trans-cellular passage of water has also been suggested by Alvarez and Yodilevich (1967) and by Faber and Hart (1967). The values obtained for the retial hydraulic conductivity indicated that the intercellular pores had diameters of about 1300 Å. Since the capillaries had a low permeability to albumin and dextran-75 000 (mol. diam. about 120 Å) the fibrillar matrix which may be observed within the junctions was assumed to behave as a filter to these molecules. Removal of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  lead to a significant increase in the capillary permeability to lipid-insoluble molecules, whereas the exchange of THO and lipid-soluble molecules remained unchanged. The primary site of the action of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  was suggested to be on the endothelial cell membrane and not on the pores (junctions). However after prolonged exposure to the chelating agent, EDTA, an increase in the permeability of high-molecular dextrans was observed. Since no changes in the junctional dimensions could be detected on electron micrographs from these *retiae*, we assumed this permeability increase to dextran to be due to changes in the structure of the fibrillar matrix of the junctions.

The present data show that the gill membrane have a permeability pattern which in many ways is similar to that of the retial capillaries. The gills were thus found to be 7 and 25 times more permeable to THO than to urea and  $^{22}\text{Na}^{+}$  respectively (Table IV). This strongly indicates that the major part of the THO-diffusion and thereby the  $\text{H}_2\text{O}$ -diffusion takes place through the endothelial cells. Similarly lipid-soluble substances such as ethanol, anipyrone and partly urea must diffuse across the cell membranes since their permeabilities are significantly higher than would be predicted from their free diffusion coefficients as compared to those of Na and Cl. The  $Q_{\text{sw}}$ -values of the permeabilities support this. Values similar to those of the free diffusion coefficients were found for  $^{22}\text{Na}^{+}$  and  $\text{Cl}^{-}$  whereas lipid-soluble substances showed higher  $Q_{\text{sw}}$ -values.

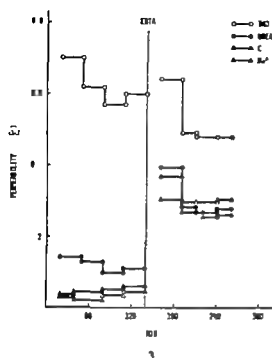


Fig. 3. The effect of EDTA. When EDTA was added to the perfusate in a concentration of about 120 of the total content of Ca and Mg in plasma, the permeability of urea, Cl<sup>-</sup> and Na<sup>+</sup> increased dramatically. The THO-permeability however appeared to remain fairly unchanged. In this experiment the gills were irrigated with water at 20°C.

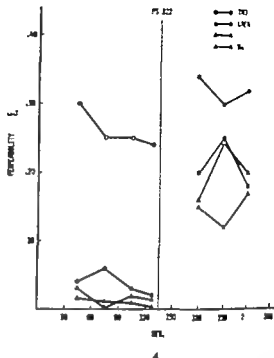


Fig. 4. The effect of MS222. The addition of 2 g/l of MS222 to the ventilate lead to a marked increase in the permeability of urea, Cl<sup>-</sup> and Na<sup>+</sup> whereas the exchange of THO was not affected. In this experiment the gills were irrigated with water at 20°C.

the perfusate activity. In this latter case release of <sup>86</sup>K from the gill tissue was shown to take place by the fact that the <sup>86</sup>K-activity was temporarily about 50% higher in the output perfusate than in the input.

The effect of temperature was also examined in gills treated with EDTA sufficient to give significant changes in permeabilities. As shown in Table V the *Q* for the test-substances (including THO and the lipid-soluble molecules) was very similar to that of the corresponding free diffusion coefficients.

#### *The effect of the local anaesthetic MS-222*

MS-222 is an isomer of benzocaine (methanesulphonate of meta-aminobenzoic acid) and is widely used as an anesthetic for aquatic animals. It is added to water in ratios between 1:1000 and 1:10000.

The effect of MS-222 (Sandoz) on the exchange was studied in 14 experiments. The anaesthetic was added either to the ventilate or to the perfusate in concentrations of 2 g/l.

During perfusions with plasma containing MS-222 no changes could be observed in the exchange of the test-substances. When the drug had been added to the ventilate, however, the effects on the permeability properties of the gill membrane were rather dramatic and

It is interesting to compare the permeability properties of the gills with those of the capillaries in the *rete* of the swimbladder. The *rete* is an internal exchange organ designed to allow rapid gas exchange between arterial and venous blood. The gills should likewise allow rapid gas exchange but at the same time limit exchange of water and ions. Such comparison shows that the *rete* is 10 times more permeable to  $K^+$  and THO than is the gill, whereas the urea-permeability is about the same in these organs (Stray-Pedersen and Steen 1975).

The diffusion coefficient of THO through the gill membrane is  $7.3 \cdot 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$ . Extrapolation to the size of water gives  $D_{H_2O} = 11 \cdot 10^{-9} \text{ cm}^2 \text{ sec}^{-1}$ . This value is about 3000 times smaller than the free diffusion coefficient for  $H_2O$  ( $\sim 2.7 \cdot 10^{-5}$  at  $25^\circ\text{C}$ ).

The proportion of the total gill area available for ion diffusion is very small. The free diffusion coefficient of  $Na^+$  in water is  $1.48 \cdot 10^{-5} \text{ cm}^2 \text{ sec}^{-1}$  whereas the  $D_{Na}$  for the gills is  $2.9 \cdot 10^{-9}$ . Provided  $Na^+$  will diffuse only through intercellular pores and without restriction then only about 1,200,000 of the gill area is available to ion diffusion. Since the area of the gills perfused in these experiments was about 200  $\text{cm}^2$  the total slit area is roughly  $0.001 \text{ cm}^2$ .

In our experiments an osmotic water flow could not be detected. Therefore data are not available to calculate the pore size based on Poiseuille's equation. However since sucrose (mol. diam. = 11 Å) appears to be greatly restricted, the pore diameter is most likely not much greater than 20 Å. In this case there would be a total of  $7 \cdot 10^6$  pores, i.e.  $3 \cdot 10^4$  pores per square centimeter of gill surface.

The fact that the gill barrier has slits of small dimensions has consequences for its osmotic behaviour. It has been appreciated for many years that the osmotic flow of water can be hundreds of times faster than the diffusional flow of water if the separating membrane has pores with diameter above 50 Å. (Pappenheimer 1953, Meisler and Setnikar 1958.) However the larger the pore the smaller will be the osmotic pressure exerted by small molecules. It appears that the slit width of the gills may be the optimal compromise between the highest possible resistance to flow and the lowest possible osmotic effect of the smallest and most abundant ions. Although further research on flow through small pores are needed, we would like to stress that the flux of water across a biological barrier must be based on data on the hydraulic conductivity as well as on the effective osmotic pressure.

The work of Durbin (1960) on osmotic flow through cellulose membranes with varying pore diameter illustrates our point. Durbin used dialysis tubing and wet gel membranes with pore diameter of 23 and 32 Å respectively. The total pore area for the two membranes were the same and the hydraulic conductivity ( $L_p$ ) was measured to be 25 and 370  $\mu\text{l}/24 \text{ atm}/\text{min}$  respectively.

He measured the flow of water across the membranes when they separated water from solutions of different solutes. Using the hydraulic conductivity he could then calculate the effective osmotic pressure difference in each case. He found that the effective osmotic pressure was progressively smaller than calculated from the van't Hoff equation the smaller the ion. This deviation is expressed by the Staverman factor ( $\sigma$ ) and expresses the "leakiness" of the membrane to each solute. Let us consider the osmotic behaviour when these membranes separate plasma from water. To simplify the discussion we assume plasma to consist of 280 meq/l "ions" 7 mM/l glucose and 1 mM/l of "proteins". Based on Durbin's

The size of the intercellular pores, must be considerably smaller in the gill membrane than in the rete, since  $^{22}\text{Na}^+$  was found to be at least 20 times as permeable as sucrose where the ratio of their free diffusion coefficients is 3. This would signal pore diameters below 20 Å. This means either that the junctions of gill membrane are actually narrower than those of the retial capillary membrane or simply reflect differences in the structure of the pore matrix. Since the osmotic (or hydrodynamic) permeability of water across the gill membrane was not measured, this point can not be ascertained.

The exchange of the test substances was the same in the direction from perfusate to ventilate as in the opposite direction. The transbranchial transport was not influenced by the presence of metabolic inhibitors such as KCN or ouabain. However clear evidence for active uptake by the gill tissue of  $\text{K}^+$  from the perfusate was obtained. According to Macdonald (1971) active transport may represent 20% of the total *influx* of  $\text{NaCl}$ . Our methods were not sufficiently accurate to detect such a difference between the exchange of  $\text{Na}^+$  or  $\text{Cl}^-$  from ventilate to perfusate and the exchange from perfusate to ventilate.

The accumulation of  $\text{K}^+$  in the gill tissue could be effectively abolished by ouabain and/or KCN but no effect on its transbranchial exchange could be detected.

The divalent ions,  $\text{Ca}^{++}$  and/or  $\text{Mg}^{++}$  play an important role in maintaining the selective permeability property of the fish gill barrier (Potts and Fleming 1970, Cuthbert and Macdonald 1971). When these ions were removed by EDTA in the present study the permeability pattern of the gills changed dramatically and in a similar way as was observed in the *retila* (Stray-Pedersen 1975). The permeabilities of ions and lipid-insoluble molecules increased by a factor of 5 to 20, whereas the permeabilities of water and lipid-soluble molecules were not affected to an extent which could be detected with certainty. After 30 min of EDTA exposure the permeability pattern but not the absolute permeabilities of the gills resembled closely that of an aqueous barrier (Table VI). The permeability ratio between  $\text{Cl}^-$  and sucrose obtained at such circumstances were about 2, the ratio between their free diffusion coefficients being about 4. The fact that dextran-75 000 did not pass the EDTA treated gills to any measurable extent indicated that severe edema or tissue damage had not occurred.

The main site of action of  $\text{Ca}^{++}/\text{Mg}^{++}$  resides most likely in the cell membranes rather than in the intercellular junctions. Removal of these ions is known to cause permeability changes in a variety of cells (Morill and Robbins 1967). If the effect of EDTA was due to widening of the pores to such an extent as to give a 70 fold increase in the  $\text{Na}^+$ -permeability then a measurable increase in the permeability of THO should be expected. This was not found. The fact that the accumulation of  $\text{K}^+$  was significantly smaller in the EDTA treated gills, indicated the cells to be more leaky to ions. Moreover in the EDTA-treated gills the  $Q_{10}$ -values of the permeabilities of the test-substances were almost similar to those of the corresponding free diffusion coefficients (Table VI), indicating that removal of  $\text{Ca}^{++}/\text{Mg}^{++}$  lead to alterations in the lipid character of the membrane.

The local anaesthetic, MS 222 elicited the same type of permeability changes as did EDTA. It is therefore reasonable to suggest the site of action of this drug also to be on the cell membranes. Since the permeability changes could only be induced when MS 222 had been added to the ventilate it is tempting to suggest that its "receptor" sites are on the epithelial cell rather than on the endothelial cell.

uptake in a 100 g eel in freshwater has been measured to be about 1  $\mu\text{l}/\text{min}$  (Evans 1969). The total pore area is 1/200 000 of the gill area, but the diffusion coefficient for water through gills is 1/3 000 of that through the pores. The diffusion of water across the pores will therefore be 1/3 000/200 000 = 0.014  $\mu\text{l}/\text{min}$ , i.e. only 1.5% of the total water diffusion occurs through the pores.

One of us (S.S.P.) has been Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council and from Norsk Værnkrigsfond is gratefully acknowledged. We also wish to thank Prof. Dr. B. A. Weiler for his valuable comments on the manuscript and Mrs. G. Ålvén for skilful technical assistance.

## References

- AL AKSE, O. A. and D. L. YODanis, Capillary permeability and tissue exchange of water and electrolytes in the stomach. *Amer J Physiol.* 1967 215 315-322.
- BACHTHAL, M. W. and T. E. RYAN, Junctions between intimately apposed cell membranes in the vertebrate brain. *J. cell. Biol.* 1968 40 643-677.
- CHAMBERLAIN, R. and B. W. ZWISLOCKI, Capillary endothelial control in relation to permeability. *J. cell. comp. Physiol.* 1960 15 255-272.
- CROOK, C., The permeability of capillaries in various organs as determined by use of the indicator diffusion method. *Acta physiol. scand.* 1963 59 293-305.
- CROOK, C., Capillary permeability—techniques and problems. In: *Capillary Permeability*. Ed. C. Crook and N. A. Lassen. Munksgaard, Copenhagen, 1970. 15-31.
- CUTRICK, A. W. and J. MAXEY, The effect of calcium and magnesium ions on sodium fluxes through gills of *Carassius auratus* L. *J. Physiol. (Lond)* 1972 221 633-643.
- DURAND, R. F. Osmotic flow of water across permeable cellulose membranes. *J. gen. Physiol.* 1960 44 315-326.
- E AND, D. H. Studies on the permeability to water of selected marine, freshwater and euryhaline teleosts. *J. exp. Biol.* 1969 50 689-703.
- FABER, J. J. and F. M. HART, Transfer of charged and uncharged molecules to the placenta of the rabbit. *Amer J Physiol.* 1967 214 890-894.
- HUGHES, H. M. The dimensions of fish gills in relation to their function. *J. exp. Biol.* 1966 43 177-195.
- HUGHES, G. M. and A. V. ORRISON, The fine structure of the secondary lamellae of the gills of *Gasterosteus aculeatus*. *Quart. J. micr. Sci.* 1965 106 343-353.
- MARTZ, J. Fish Gills. Mechanisms of salt transfer in fresh water and sea water. *Phil. Trans. R.* 1971. 262 209-249.
- MERCLA, G. and I. SETNBAR, Experimental study of osmosis through cellulose membranes. *J. gen. Physiol.* 1958 42 429-444.
- MORELL, G. A. and E. ROBERTS, The role of calcium in the regulation of the steady-state levels of sodium and potassium in the HeLa cell. *J. gen. Physiol.* 1967 50 781-792.
- MOTAN, R. and F. GARCIA-RODRIGUEZ, Transport mechanisms in the teleostean gill and amphibian skin. *Amer. Rev. Physiol.* 1972 54 141-176.
- NICOLAYSEN, G., Extravascular concentrations of calcium and magnesium ions and edema formation in selected lungs. *Acta physiol. scand.* 1971 a. 81 325-339.
- NICOLAYSEN, G., Increase in capillary filtration rate resulting from reduction in the extravascular calcium ion-concentration. *Acta physiol. scand.* 1971 b. 81 517-527.
- PAPPENHIMER, J. R., Passage of molecules through capillary walls. *Physiol. Rev.* 1951. 31 387-423.
- PAPPENHIMER, J. R., E. M. RYCKOFF and L. M. BORRERO, Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *Amer J Physiol.* 1951 167 13-44.
- PORTER, W. T. W. and W. R. FLETCHER, The effects of prostatic and divalent cations on the permeability to water of *Fundulus heteroclitus*. *J. exp. Biol.* 1970. 53 317-327.
- STEVENS, J. B. The physiology of the water bladder in the eel, *Anguilla vulgaris* L. II. The reabsorption of water. *Acta physiol. scand.* 1963 35 138-149.

TABLE VII Staverman factor osmotic pressure and osmotic flow for two synthetic membranes separating plasma from water (See text for details.)

Concentration in "plasma"	Staverman factor ( $\sigma$ )		Osmotic pressure ( $\pi$ ) $\pi = \sigma \Delta C \cdot 24 \cdot 10^{-3}$ (atm)		Osmotic flow (V) $V = \pi L_p$ ( $\mu$ l/min)	
	Dialysis tubing	Wet gel	Dialysis tubing	Wet gel	Dialysis tubing	Wet gel
280 meq/l ions	0.01	0.002	0.067	0.0134	0.125	0.5
7 mM/l glucose	0.20	0.016	0.034	0.003		
1 mM/l protein	1.0	0.73	0.04	0.0184		

data on the Staverman factor for different substances we assess reasonable values for each of these three substance categories (Table VII). We can now calculate the osmotic pressure exerted and the resulting water flow for each of the three components across both membranes. The osmotic pressure is

$$\pi = \Delta C \cdot \sigma \cdot 24 \cdot 10^{-3} \text{ atm}$$

since a  $\Delta C$  of 1 mM/l causes an osmotic pressure of  $24 \cdot 10^{-3}$  atm. Table VII compares the calculated values. We shall notice that while van t Hoff's equation predicts plasma ( $\Delta C$  300 mOsm/l) to exert an osmotic pressure of 7.2 atm, the porosity reduces this to 0.125 for the dialysis tubing and 0.035 atm for the wet gel.

This osmotic pressure will act to such water across the gill barrier and into the plasma. The capillary pressure will act in the opposite direction. The mean branchial blood pressure in the eel is about 40 cm H<sub>2</sub>O whereas the osmotic pressure across the two cellulose membranes of Durbin was 126 and 35 cm H<sub>2</sub>O respectively. This suggests that if the intercellular pores of the gill really have widths of about 20 Å, then the blood pressure in the gill capillaries may be of significance in reducing the osmotic water uptake in freshwater fishes.

Finally we can estimate the proportion of the total water diffusion which occurs through the pores.

The total diffusional water flux across the gills is equal to

$$\dot{Q} = D \frac{A \Delta C}{l}$$

Where

$\dot{Q}$  = net diffusion of water from water to plasma in cm<sup>3</sup>/min.

$D$  = the diffusion coefficient =  $8.0 \cdot 10^{-9}$  cm<sup>2</sup>/sec =  $4.8 \cdot 10^{-4}$  cm<sup>2</sup>/min

$A$  = gill area = 200 cm<sup>2</sup>

$\Delta C$  = difference in water concentration between plasma and ventilation water  
= 300 mM/l = 0.3 mM/cm

$l$  = thickness of barrier =  $5 \mu$  =  $5 \cdot 10^{-4}$  cm

Using these values  $\dot{Q}$  becomes  $5 \cdot 10^{-3}$  mM/min. Since 1 mM of water occupies 18  $\mu$ l the total rate of water diffusion becomes 0.9  $\mu$ l/min. This indicates that osmotic water uptake may not be more important than diffusional water uptake of water since the total water

uptake in a 100 g eel in freshwater has been measured to be about  $1 \mu\text{l}/\text{min}$  (Evans 1969). The total pore area is  $1/200\,000$  of the gill area, but the diffusion coefficient for water through gills is  $1/3\,000$  of that through the pores. The diffusion of water across the pores will therefore be  $1/1\,3000/200\,000 = 0.014 \mu\text{l}/\text{min}$ , i.e. only 1.5% of the total water diffusion occurs through the pores.

One of us (S.S.P.) has been Research Fellow of the Norwegian Research Council for Science and the Humanities. Financial support from this Council and from Norsk Vannfriskelund is gratefully acknowledged. We also wish to thank Prof. Dr. B. A. Waaler for his valuable comments on the manuscript and Mrs. G. Ålsvær for skilful technical assistance.

## References

- AL ARIDJ, O. A. and D. L. YUDELEVICH, Capillary permeability and tissue exchange of water and electrolytes in the stomach. *Amer. J. Physiol.* 1967 213 315-322.
- BENNETT, H. W. and T. S. REIER, Junctions between ultrathin apposed cell membranes in the vertebrate brain. *J. cell. Biol.* 1969 42 644-677.
- CHAMBERS, R. and B. W. ZWITZACH, Capillary endothelial contact in relation to permeability. *J. cell. comp. Physiol.* 1960 15 255-272.
- CROOS, C. The permeability of capillaries in various organs as determined by use of the "indicator diffusion" method. *Acta physiol. scand.* 1963 38 292-305.
- CROOS, C., Capillary permeability—techniques and problems. In: *Capillary Permeability* Ed. C. Croos and N. A. Lassen. Munksgaard, Copenhagen, 1970 15-31.
- CUTLER, A. W. and J. MARZ, The effect of calcium and magnesium ions on sodium fluxes through gills of *Carassius auratus* L. *J. Physiol. (Lond.)* 1972 221 633-643.
- DURAN, R. F. Osmotic flow of water across permeable cellulose membranes. *J. gen. Physiol.* 1960 44 315-326.
- EVANS, D. H., Studies on the permeability to water of selected sparrow, freshwater and euryhaline teleosts. *J. exp. Biol.* 1969 30 689-703.
- FARIS, J. J. and F. M. HART Transfer of charged and uncharged molecules in the placenta of the rabbit. *Amer. J. Physiol.* 1967 213 890-894.
- FRUMER, G. M., The dimensions of fish gills in relation to their function. *J. exp. Biol.* 1966 45 177-193.
- FRUMER, G. M. and A. V. GARTWORT, The fine structure of the secondary lamellae of the gills of *Gasterosteus aculeatus*. *Quart. J. micr. Sci.* 1965 106 343-353.
- MARZ, J. Fish Gills, Mechanisms of salt transfer in fresh water and sea water. *Phil. Trans. R.* 1971 262 209-249.
- MEKILÄ, O. and I. SETälÄN, Experimental study of osmosis through cellulose membranes. *J. gen. Physiol.* 1958 42 429-444.
- MORRILL, G. A. and E. ROBERTS, The role of calcium in the regulation of the steady-state levels of sodium and potassium in the HeLa cell. *J. gen. Physiol.* 1967 50 781-792.
- MOTAN, R. and F. GARCIA-RODRIGUEZ, Transport mechanisms in the teleostean gill and nephrocytic cells. *Amer. Rev. Physiol.* 1972 34 141-176.
- NICOLAYSEN, G., Intravascular concentrations of calcium and magnesium ions and edema formation in isolated lungs. *Acta physiol. scand.* 1971 a 81 325-339.
- NICOLAYSEN, G. Increase in capillary filtration rate resulting from reduction in the intravascular calcium ion-concentration. *Acta physiol. scand.* 1971 b 81 517-527.
- PAPPENHIMER, J. R., Passage of molecules through capillary walls. *Physiol. Rev.* 1953 33 387-423.
- PAPPENHIMER, J. R., E. M. REIDEN and L. M. BORRERO, Filtration, diffusion and molecular sieving through peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *Amer. J. Physiol.* 1951 167 13-46.
- POTTS, W. T. W. and W. R. FLEMING, The effects of protease and divalent cations on the permeability to water of *Fundulus heteroclitus*. *J. exp. Biol.* 1970 55 317-327.
- STEVENS, J. B. The physiology of the swimbladder in the eel, *Anguilla rostrata*. II. The reabsorption of gases. *Acta physiol. scand.* 1965 33 138-149.



TABLE I Effect of 60 min cold exposure in hydropenic man

	GFR ml/min $\pm$ 73 m <sup>2</sup>			RPF ml/min $\times$ 1.73 m <sup>2</sup>			$C_{\text{osm}}/C_{\text{In}}$ %			$C_{\text{Na}}/C_{\text{In}}$ %		
	a	b	c	a	b	c	a	b	c	a	b	c
Mean	107	111	106	603	533	561	2.39	2.67	2.85	1.11	1.38	1.54
S.D.	19.35	27.39	26.91	163.5	143.7	191.8	0.47	0.55	0.48	0.29	0.43	0.41
p		>0.05	>0.05		>0.05	>0.05		<0.05	<0.01		<0.05	<0.01

Effect on glomerular filtration rate, renal plasma flow, fractional excretion of osmoles, fractional excretion of sodium. Individual data, means and standard deviations from 9 healthy subjects. a = means of 3 30-min control periods at room temperature; b = 0–30 min in cold; c = 30–60 min in cold; p = values are calculated by paired observations.

The hydropenic state was effected by the withdrawal of all liquid and solid nourishment for 18 h prior to the study. Hydropenic state was checked in the laboratory by 3 consecutive 30 min urine samples which had to show stable flow and an osmolality of  $>1000$  mOsm/kg  $\text{H}_2\text{O}$ .

Standard clearance technique was used. Glomerular filtration rate (GFR) was measured as the clearance of inulin and renal plasma flow (RPF) as the clearance of PAH, both in ml/min and  $\pm 73$  m<sup>2</sup> body surface. 0.5 ml/kg of a solution consisting of 85 mg/ml of inulin (Lacvoan Gesellschaft) and 30 mg/ml of para-aminohippuric acid (PAH) (Merck, Sharp & Dohme) was given as a prime dose. The solution was then infused by a motor-driven syringe at a constant rate of 0.5 ml/min. The clearance infusion was allowed to equilibrate for 90 min.

The bladder was catheterized with a double lumen catheter and emptied at the end of each period by air insufflation, vacuum suction and gentle suprapubic compression. To ensure a complete emptying, the bladder was rinsed with 20 ml of sterile water at the end of each period.

Mid period blood samples were drawn from a cubital vein. Arterial systolic blood pressure was recorded manually from one arm by mercury manometer.

The volunteers were naked and covered with blankets. They spent three control periods of 30 min at room temperature. Then the blankets were removed and the temperature on the table was lowered to  $+15^\circ\text{C}$ . All experiments were terminated by two periods of 30 min in cold.

Standard statistical methods were used including paired observations and Student's *t* test. All values are given as means  $\pm$  standard deviation.

#### Analyses

In blood and urine samples, sodium was analyzed in a flame photometer (IL Model 143) and the osmolality was analyzed cryoscopically in a Knauer osmometer. Inulin was analyzed according to Heyrovsky (1956) and PAH according to Brum (1951).

Tubular reabsorption of solute free water ( $T^0_{\text{osm}}$ ) was calculated from the expression  $(C_{\text{osm}} - V) / V$ , where  $V$  = urine flow in ml/min and  $C_{\text{osm}}$  = clearance of osmoles in ml/min.

## Results

Individual data from 9 hydropenic subjects are listed in Table I and II.

#### Effect on renal hemodynamics

Exposure to cold for 1 h at  $+15^\circ\text{C}$  did not influence glomerular filtration rate. At room temperature, mean GFR was  $107 \pm 19.4$  ml/min and  $\pm 73$  m<sup>2</sup> body surface and after 60 min of cold  $106 \pm 26.9$  ml/min and  $\pm 73$  m<sup>2</sup> body surface ( $p > 0.05$ ) (Table I).

During cold exposure renal plasma flow changed from  $603 \pm 163.5$  ml/min and  $\pm 73$  m<sup>2</sup> body surface at room temperature to  $561 \pm 191.8$  ml/min and  $\pm 73$  m<sup>2</sup> body surface but this change did not differ statistically from controls ( $p > 0.05$ ) (Table I).

TABLE II. Effect of 60 minutes cold exposure in hydropenic man

	Arterial BP mm Hg			$T_{H_2O}^*/C_{H_2O}$ %			$U_{H_2O}/C_{H_2O}$ %		
	b			b			b		
Mean	116	131	133	1.68	1.89	2.00	0.72	0.78	0.85
S.D.	9.0	11.6	10.0	0.31	0.33	0.32	0.2	0.2	0.2
p		0.001	<0.001		<0.05	<0.01		>0.05	<0.05

\*Effect on arterial blood pressure, free water reabsorption and urinary flow. For details see Table I.

#### Effect on renal excretion of osmoles and sodium

The fractional excretion of osmoles ( $C_{H_2O}/C_{H_2O}$ ) increased significantly after cold exposure as did the fractional sodium excretion ( $C_{Na}/C_{H_2O}$ ). Mean osmolar excretion increased from  $2.39 \pm 0.47$  to  $2.85 \pm 0.48$  per cent ( $p < 0.01$ ) and mean sodium excretion from  $1.11 \pm 0.29$  to  $1.56 \pm 0.43$  per cent ( $p = 0.01$ ) (Table I).

#### Effect on blood pressure, free water reabsorption and urinary flow

Arterial blood pressure rose during cold from a mean of  $116 \pm 9$  mm Hg at room temperature to  $131 \pm 11.6$  after 30 min in cold and to  $133 \pm 10$  mm Hg after 60 min in cold. The increase was significant in both periods ( $p < 0.001$ ) (Table II).

Free water reabsorption, correlated to glomerular filtration rate ( $T_{H_2O}^*/C_{H_2O}$ ), increased significantly during cold exposure from a mean at room temperature of  $1.68 \pm 0.31$  to  $2.00 \pm 0.32$  per cent ( $p = 0.01$ ) (Table II).

Already after 30 min in cold, there was a small, but significant rise in  $T_{H_2O}^*$ . Fig. 1 shows the relationship between the rise in free water reabsorption and the rise in excretion of osmoles during 60 min of cold exposure.

In the hydropenic state, mean urinary flow was rather small and stable during control periods and cold exposure. At room temperature, mean urinary flow correlated to the filtration ( $U_{H_2O}/C_{H_2O}$ ) amounted to  $0.72 \pm 0.2$  per cent. It showed a slight, but significant increase to  $0.85 \pm 0.2$  per cent after 60 min in cold ( $p = 0.05$ ) (Table II).

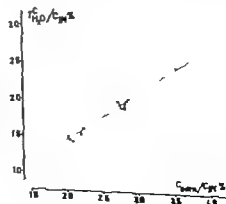


Fig. 1 Relationship between free water reabsorption ( $T_{H_2O}^*$ ) and excretion of osmoles ( $C_{H_2O}$ ) both correlated to the filtration rate in 9 hydropenic subjects during 60 min cold exposure. Each subject is represented by 5 dots, one in each of 3 control periods at room temperature, one after 30 min in cold and one after 60 min in cold. The regression line is expressed by the equation  $y = 0.08 + 0.67x$  ( $p = 0.001$ ).

## Discussion

The present results include the finding that cold exposure in man caused an increased urinary excretion of osmoles. Since this excretion was not accounted for by changes in GFR and since the load of osmoles was not changed during the study the increased excretion of osmoles was most likely due to a reduced net tubular reabsorption.

Sodium is considered to be reabsorbed without water in the ascending limb of the loop of Henle. This creates a hypertonic medullary interstitium which in the presence of ADH contributes to the concentration of the final urine by reabsorbing water from the collecting ducts (Pitts 1970). The more sodium actively pumped out of the ascending limb of the loop the higher osmotic gradient will move water from the collecting ducts. Thus, in hydropenia free water reabsorption,  $T^{\circ}_{H_2O}$  can be used as an index of sodium reabsorption by the ascending limb of the loop of Henle.

Increased  $T^{\circ}_{H_2O}$  in connection with increasing clearance of osmoles is shown by seven authors. Goldberg *et al* (1965) reported that during hypertonic saline diuresis in hydropenic man,  $T^{\circ}_{H_2O}$  increased progressively even when osmolar clearance was exceeding 27 ml/min and 1.73 m<sup>2</sup> body surface. During mannitol diuresis however  $T^{\circ}_{H_2O}$  was shown to rise to an upper limit above which the curve levelled off. This took place when the clearance of osmoles exceeded 12 ml/min and 1.73 m<sup>2</sup> body surface. In hydropenic dogs, undergoing hypertonic mannitol diuresis, Earley *et al* (1961) found this maximum in  $T^{\circ}_{H_2O}$  to occur at an osmolar clearance of 12-15 ml/min.

In a previous report from this laboratory it was suggested that tubular reabsorption of sodium during cold was related to increases in arterial blood pressure (Wallenberg and Granberg 1974). Aperia *et al* (1971) when increasing the clearance of osmoles as a function of renal artery perfusion pressure in hydropenic dogs, found the maximum in  $T^{\circ}_{H_2O}$  to correspond to an osmolar clearance of 7 ml/min/100 ml GFR, representing a perfusion pressure of more than 180 mm Hg. In the present study there was a significant linear relationship between free water reabsorption and clearance of osmoles. Clearance of osmoles however did not exceed 4 ml/min/100 ml GFR the arterial pressure did not exceed 150 mm Hg and the curve did not show any declination.

In previous studies performed in water diuresis, we did not find any measurable increase in urinary flow during cold exposure (Atterhög *et al* 1975, Wallenberg and Granberg 1974). The present results showed a small, but significant increase in urinary flow after 60 min of cold. If this represents a true difference in hydropenic state compared to water diuresis is an open question.

Previous findings indicated that the reabsorption of sodium by the distal tubules was important for the cold-induced excretion of sodium in water loaded man (Wallenberg 1974). The excretion of sodium was shown to be inhibited by infusion of hyperoncotic albumin solution during cold exposure. Parallel to this inhibition, the albumin infusion correspondingly improved fractional distal reabsorption of sodium. This result was in accordance with the view that a cold-induced disturbance in the balance between hydrostatic and oncotic pressures in peritubular capillaries might have negatively affected sodium reabsorption in the diluting segment of distal tubules.

In the present study the results showed a small, but significant increase in free water reabsorption during cold, despite a significant rise in arterial perfusion pressure. It is presumed that reduction in tubular reabsorption of sodium is dependent of increases in arterial pressure in the kidney. Obviously this is not true for the ascending limb of the loop of Henle within the small range of pressure rise produced by cold. This is supported by the findings of Aperia *et al.* (1971) showing no reduction in  $T^*_{NaO}$  in hydrophenic dogs until a perfusion pressure in the kidney above 180 mm Hg.

The parallel increase in osmolar clearance and in free water reabsorption would speak in favour to an increased concentration of osmoles entering Henle's loop during cold. The results does not exclude the increased  $T^*_{NaO}$  to be due to a reduced reabsorption of sodium by the proximal tubules. However with the methods used in this study it is not possible to directly evaluate the effect of cold exposure on different sites of the nephron.

### References

- APERIA, A. C., C. G. O. BEAUSOLEIL and E. SÖDERLUND, Relationship between renal artery perfusion pressure and tubular sodium reabsorption. *Amer. J. Physiol.* 1971 220: 1205-1212.
- ATTENBORO, J. H., P. CARLÉN, P. O. GRANBERG and L. R. WALLINBERG, Cardiovascular and renal responses to acute cold exposure in waterloaded man. *Scand. J. clin. Lab. Invest.* 1973, 33 in press.
- BAUM, C., A rapid method for the determination of para-aminohippuric acid in kidney function tests. *J. Lab. clin. Med.* 1951 37: 955-958.
- EARLEY, L. E., M. KRAM and J. ORLOFF, The effects of infusions of chlorothalidate on urinary dilution and concentration in the dog. *J. clin. Invest.* 1961 40: 857-866.
- EARLEY, L. E., I. A. MARTINO and R. M. FRIEDLER, Factors affecting sodium reabsorption by the proximal tubule as determined during blockade of distal sodium reabsorption. *J. clin. Invest.* 1966 45: 1668-1684.
- GOLDSTEIN, M. B. K. MCCORMY and M. A. RAMIREZ, Differences between saline and mannitol diuresis in hydrophenic man. *J. clin. Invest.* 1963 44: 182-192.
- HAYBOVSKY, A., A new method for the determination of uremia in plasma and urine. *Clin. Chim. Acta.* 1956, 1: 470-474.
- PITTE, R. F. Physiology of the kidneys and body fluids. *Year Book Med. Publ., Chicago.* 1970. Sec. Ed. Chapt. 7.
- WALLINBERG, L. R., Reduction in cold-induced natriuresis following hyperosmotic albumin infusion in man undergoing water diuresis. *Scand. J. clin. Lab. Invest.* 1974, 34: 233-239.
- WALLINBERG, L. R. and P. O. GRANBERG, The relationship between cold-induced natriuresis and arterial blood pressure in man. *Scand. J. clin. Lab. Invest.* 1974 34: 225-231.

## Potentiation by Prostaglandins $E_1$ , $E_2$ , and $F_2$ of the Contraction Response to Transmural Stimulation in the Bovine Iris Sphincter Muscle

By

LARS GUSTAFSSON, PER HEDQVIST and HUGO LAGERCRANTZ

Received 5 February 1975

---

### Abstract

GUSTAFSSON, L., P. HEDQVIST and H. LAGERCRANTZ. *Potentiation by prostaglandins  $E_1$  and  $F_2$  of the contraction response to transmural stimulation in the bovine iris sphincter muscle* Acta physiol. scand. 1975 95: 26-33

The contraction response to transmural stimulation of the bovine iris sphincter muscle was abolished by atropine but was left virtually unchanged by hexamethonium, and by adrenergic neuron and receptor blockers, indicating activation of postganglionic cholinergic nerve fibres. Low doses of prostaglandins  $E_1$ ,  $E_2$  and  $F_2$  ( $0.1-5 \mu\text{g/ml}$ ) consistently and apparently in a dose-dependent manner enhanced the contraction response to transmural stimulation. Prostaglandins  $E_1$  and  $E_2$  were equipotent while  $F_2$  was at least 10 times less active. Within the same dose range the prostaglandins also enhanced the contraction response to exogenous acetylcholine, indicating that part of the enhancing effect of the prostaglandins on neuromuscular transmission was due to a postjunctional action. A spontaneous increase in tone of the preparation commonly occurred about 1 h after the beginning of the experiment. SC 19,283, a potent antagonist of prostaglandin action, reduced the tone and the contraction responses as well as the enhancing effect of the prostaglandins. It is concluded that prostaglandins might serve the function of controlling neuroeffector transmission and muscular tone in the sphincter muscle of the bovine iris.

---

Prostaglandins of the E and F series (PGE, PGF) seem to be present in the iris of the rabbit, cat, sheep and cattle (Änggård and Samuelsson 1964, Ambache *et al.* 1966, Ambache and Brummer 1968, Posner 1970). The iris contains the enzyme necessary for conversion of eicosatrienoic acid into PGs (van Dorp *et al.* 1967). Mechanical irritation of the eye causes increased formation and release of PGs, as does electrical stimulation of the iris (Ambache *et al.* 1965, Posner 1970, 1973). Irritation of the eye causes miosis, increased intraocular pressure and increased protein content of the aqueous humour—effects which can be mimicked by local application of PGs (Waltzman and King 1967, Beitch and Eakins 1969).

PGs are potent inhibitors of sympathetic neuroeffector transmission and locally formed PGs have been postulated to negatively feed-back control the release of adrenergic transmitter (Hedqvist 1970, 1973). PGEs have also been claimed to influence parasympathetic

uroeffector transmission since  $\text{PGE}_1$  inhibits the effect of vagal nerve stimulation in the bovine heart (Wennum and Hedqvist 1971). Similarly  $\text{PGE}_1$  inhibits gastric secretion induced by vagal stimulation in the rat (Shaw and Ramwell 1968). On the other hand, part of the stimulant action of  $\text{PGE}_1$  on the guinea pig ileum as well as that of  $\text{PGF}_{2\alpha}$  on saliva secretion from canine submandibular glands have been suggested to be mediated via stimulation of cholinergic fibres (Horton 1965, Hahn and Patil 1972, 1974).

In the present paper are reported some actions of PGs on contraction response to parasympathetic nerve stimulation in the sphincter muscle of the bovine iris. A preliminary account of the results has been presented elsewhere (Gustafson, Hedqvist and Lagerkvist 1973).

### Methods

Ovine eyes were enucleated 5-10 min post mortem, immediately placed in ice-cold Tyrode and used within 4 h. Two strips of the sphincter muscle, with an approximate length of 15 mm were dissected free from the upper and lower margins of the iris. The preparation was mounted on 5 ml organ baths and connected to a motorized mechanical transducer (Harvard Inst. Inc.) with a load of 2-9.4 g. Changes in basal tone as well as induced contractions of the preparation were recorded on Grass model 5 Polygraph or Jeannotte electronic writer. Replacing the isotonic transducer by force displacement strain gauge transducer (Grass FT03) gave constant recordings. As bath fluid was used either Tyrode (concentrations in mM): NaCl 136.9, KCl 2.7,  $\text{CaCl}_2$  1.8,  $\text{MgCl}_2$  1.0,  $\text{NaHCO}_3$  11.9,  $\text{NaH}_2\text{PO}_4$  0.3, glucose 4.5, or Krebs solution (concentrations in mM): NaCl 120, KCl 3.9,  $\text{CaCl}_2$  2.5,  $\text{MgCl}_2$  1.2,  $\text{NaHCO}_3$  15.5,  $\text{NaH}_2\text{PO}_4$  1.2, glucose 11.5. The bath fluid was kept at 37°C and was gassed with 5%  $\text{CO}_2$  in  $\text{O}_2$ . The preparation was stimulated through platinum electrodes along the wall of the bath and a Grass S4 stimulator delivering trains of biphasic pulses (1-20 Hz, 1 ms duration, 15-25 V effective strength, 1-10 pulses) at 1 min intervals. A standard type of stimulation (3 Hz, 15 pulses) was routinely used unless otherwise stated.

The following compounds were used: acetylcholine chloride (SIGMA Chemicals, St. Louis, U.S.A.), atropine sulfate (SIGMA), ginsenosides sulfate (Ciba-Geigy Basel, Switzerland), isoproterenol bromide (SIGMA), histamine chloride (ACD, Sweden), 1-noradrenaline hydrochloride (SIGMA), phenylephrine methanesulfonate (Ciba-Geigy), propranolol hydrochloride (ICI Ltd., Great Britain), prostaglandin  $\text{E}_2$ ,  $\text{E}_1$  and  $\text{F}_{2\alpha}$  as free acids (kindly supplied by Dr J. Pike, The Upjohn Co., Kalamazoo, Mich., U.S.A.) and 8C 14220 (1-acetyl-2,3-dichloro-10,11-dihydrobenzo (a, b) (1,4) oxazepine-10-carboxyl hydrazine), kindly supplied by Dr J. H. Sauer, The G. D. Searle and Co., Chicago, Ill., U.S.A.).

### Results

When the isolated bovine iris sphincter muscle was mounted in the organ bath, it remained at its resting length (15-17 mm) for approximately 1 h whereafter it slowly began to contract. This occurred independently of whether the preparation was stimulated or not. In preparations which reached a stable high tone the final length was 4-5 mm. Although the spontaneous increase in basal tone could be partially counteracted by repeated washing of the preparation, experiments were always carried out during the first hour after mounting the preparation unless otherwise stated.

Transverse stimulation (3 Hz, 15 pulses) of the sphincter muscle produced a rapid and marked contraction which in some preparations was followed by a poststimulatory relaxation below the resting level. Single pulses were also sufficient to induce a contraction. The contraction induced by transverse stimulation could be mimicked by acetylcholine (ACh) 0.4  $\mu\text{g/ml}$ , and was completely abolished by atropine, 0.15  $\mu\text{g/ml}$ . The relaxation compo-

## Potentiation by Prostaglandins $E_1$ , $E_2$ , and $F_2$ of the Contraction Response to Transmural Stimulation in the Bovine Iris Sphincter Muscle

By

LARS GUSTAFSSON, PER HEDQVIST and HUGO LAGERCRANTZ

Received 5 February 1975

---

### Abstract

GUSTAFSSON, L., P. HEDQVIST and H. LAGERCRANTZ. *Potentiation by prostaglandins  $E_1$  and  $F_{2\alpha}$  of the contraction response to transmural stimulation in the bovine iris sphincter muscle* Acta physiol scand, 1975, 95, 26-33

The contraction response to transmural stimulation of the bovine iris sphincter muscle was blocked by atropine but was left virtually unchanged by hexamethonium, and by adrenergic neuron and receptor blockers, indicating activation of postganglionic cholinergic nerve fibres. Low doses of prostaglandins  $E_1$ ,  $E_2$  and  $F_{2\alpha}$  (0.1-3.0 ng/ml) consistently and apparently in a dose-dependent manner enhanced the contraction response to transmural stimulation. Prostaglandins  $E_1$  and  $E_2$  were equipotent, while  $F_{2\alpha}$  was at least 10 times less active. Within the same dose range the prostaglandins also enhanced the contraction response to exogenous acetylcholine, indicating that part of the enhancing effect of the prostaglandins on neuromuscular transmission was due to a postjunctional action. A spontaneous increase in tone of the preparation commonly occurred about 1 h after the beginning of the experiment. SC 19220, a potent antagonist of prostaglandin action, reduced the tone and the contraction responses as well as the enhancing effect of the prostaglandins. It is concluded that prostaglandins might serve the function of controlling neuroeffector transmission and muscular tone in the sphincter muscle of the bovine iris.

---

Prostaglandins of the H and F series (PGE, PGF) seem to be present in the iris of the rabbit, cat, sheep and cattle (Ånggård and Samuelsson 1964, Ambache *et al* 1966, Ambache and Brummer 1968, Posner 1970). The iris contains the enzyme necessary for conversion of eicosatrienoic acid into PGs (van Dorp *et al* 1967). Mechanical irritation of the eye causes increased formation and release of PGs, as does electrical stimulation of the iris (Ambache *et al* 1965, Posner 1970, 1973). Irritation of the eye causes miosis, increased intraocular pressure and increased protein content of the aqueous humour, effects which can be mimicked by local application of PGs (Waltzman and King 1967, Beitch and Eakins 1969).

PGs are potent inhibitors of sympathetic neuroeffector transmission and locally formed PGs have been postulated to negatively feed-back control the release of adrenergic transmitter (Hedqvist 1970, 1973). PGEs have also been claimed to influence parasympathetic

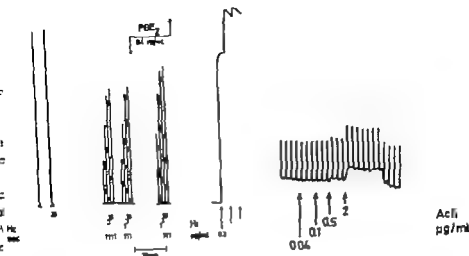


Fig. 3. Isolated bovine iris sphincter muscle. Submaximal contraction responses to 1 sec pulse trains at 3, 10, and 20 Hz shown to be potentiated by addition of  $PGE_2$ . To the left and right maximal amplitude of contraction responses to respectively transmuscular stimulation (20 Hz for 5 and 10 sec) and histamine.

Fig. 4. Isolated bovine iris sphincter muscle. Effect of subpotent agonistic and spasmogenic doses of acetylcholine on contraction responses to transmuscular stimulation (20 Hz 3 pulses, 1 sec) at 1 min intervals.

$PGE_2$  enhanced the contraction response to transmuscular stimulation at 1, 3, 10, and 20 Hz, provided the stimulation period was kept short (1 s) (Fig. 3). When the preparation was stimulated at 20 Hz for 5 or 10 s a near maximal contraction occurred, as witnessed also by the contraction responses to histamine, and  $PGE_2$  did not significantly alter the response. In Table I is summarized the effect of 2 doses of  $PGE_2$  on the contraction response to transmuscular stimulation at different stimulation frequencies. It can be seen that, although  $PGE_2$  significantly enhanced the contraction response at all frequencies used, the effect decreased with increasing stimulation frequency.

In 13 other experiments it was investigated whether acetylcholine, as reported above for

TABLE I. Isolated bovine iris sphincter muscle. Contraction response to 1 periods of transmuscular stimulation at 1, 3, 10 and 20 Hz, 10 min after addition of  $PGE_2$ , compared to that obtained during preceding corresponding control stimulation. Number of experiments. Statistical analysis according to Student's *t*-test for paired and unpaired data ( $-p < 0.05$ ,  $-p < 0.01$ ).

$PGE_2$ µg/ml	Stim. freq.	Responses in % of control		
		Mean	$\pm$ S.E.	<i>p</i>
0.1	1	134.4	10.2	.01
0.2	3	137.3	8.7	.01
0.2	10	131.4	6.6	.01
0.2	20	126.7	4.3	.01
0.4	1	179.0	14.3	.001
0.4	3	149.0	9.2	.01
0.4	10	131.6	5.0	.001
0.4	20	125.6	4.5	.001



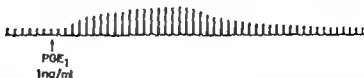


Fig. 1 Isolated bovine iris sphincter muscle. Effect of  $\text{PGE}_1$  (1 ng/ml) on contraction responses to transmural stimulation (3 Hz, 15 pulses, 1 ms, supramaximal voltage) at 1 min intervals. Wash as det.

ment which was regularly observed after atropine administration could be blocked by guanethidine, 0.8  $\mu\text{g/ml}$  or propranolol 20  $\mu\text{g/ml}$ . In these doses guanethidine or propranolol did not alter the contraction response to transmural stimulation. In a 10 times higher dose guanethidine produced an inhibition of induced contractions (cf. Boura and Ger 1965). Hexamethonium, 5–20  $\mu\text{g/ml}$ , or phentolamine, 4  $\mu\text{g/ml}$ , had no effect on contraction response to transmural stimulation. Administration of noradrenaline (NA), 0.2  $\mu\text{g}$ , relaxed the preparation, an effect which was altered to contraction when propranolol 20  $\mu\text{g/ml}$  had been given in advance.

Addition to the bath of  $\text{PGE}_1$  in doses ranging from 0.2–10 ng/ml consistently and dose dependently increased the contractile response to transmural stimulation (3 Hz, 15 pulses) (Fig. 1). Lower doses (down to 1  $\mu\text{g/ml}$ ) did not alter responses while higher doses (10 ng/ml) caused a direct contraction of the preparation without further increasing the response to transmural stimulation. The potentiation by  $\text{PGE}_1$  was visible within 60 s, reached a maximum after 5–10 min and remained at this level for at least another 10 min poststimulation. After washing the preparation the responses returned to normal over a period of 5–10 min.

$\text{PGE}_2$  produced qualitatively and quantitatively the same effects as  $\text{PGE}_1$  on the sphincter muscle, whereas  $\text{PGF}_{2\alpha}$  was found to possess a much weaker stimulant effect on the contraction response to transmural stimulation. A summary of the effects of different doses of  $\text{PGE}_1$ ,  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$  is given in Fig. 2.

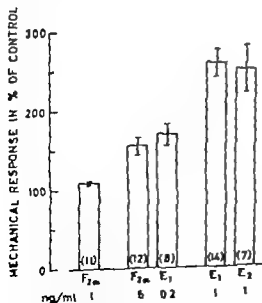


Fig. 2. Isolated bovine iris sphincter muscle. Comparison of enhancing effect on contraction response to transmural stimulation by  $\text{PGE}_1$ ,  $\text{PGE}_2$  and  $\text{PGF}_{2\alpha}$ . Stimulus parameters as in Fig. 1. Response measured immediately before (control) and 6 min after addition of respective prostaglandin. Mean values  $\pm$  S.E. Figures within brackets = number of expts.

- TERERA, S. H., A. HERMAN and J. R. VANE, Prostaglandin generation maintains the smooth muscle tone of the rabbit isolated jejunum. *Brit. J. Pharmacol.* 1972. 44 329-330.
- UNTHAM, L. E., B. HEDQVIST and H. LAGERCRANTZ, Prostaglandin mediated enhancement of effector response to cholinergic nerve stimulation. *Acta physiol. scand.* 1973. Suppl. 396. 106.
- WOL, R. A. and P. M. P. TH, Salivation induced by prostaglandin  $F_{2\alpha}$  and modification of the response by atropine and physostigmine. *Brit. J. Pharmacol.* 1972. 44 527-533.
- WOL, R. A. and P. M. P. TH, Further observations on the interaction of  $PGF_{2\alpha}$  with cholinergic efferent nerves in canine salivary glands. *Eur. J. Pharmacol.* 1974. 23 279-284.
- WRIGHT, P. Studies of the effect of prostaglandin  $E_1$  and  $E_2$  on the sympathetic neuromuscular transmission in some animal tissues. *Acta physiol. scand.* 1970. 79 Suppl. 345. 1-40.
- WRIGHT, P. Prostaglandin mediated control of sympathetic neuroeffector transmission. *Adv. Biosc.* 1973. 9 461-473.
- YERGEN, E. W. Biological activities of pure prostaglandins. *Experientia (Basel)* 1965. 21 113-118.
- TH, P. M., Adrenergic receptors of the bovine iris sphincter. *J. Pharmacol. exp. Ther.* 1969 166. 299-307.
- OWEN, J. The release of prostaglandin  $E_2$  from the bovine iris. *Brit. J. Pharmacol.* 1970. 40. 163P 164P.
- OWEN, J. The action of prostaglandins on the bovine sphincter pupillae. *J. Physiol. (Lond.)* 1971. 227 257-267.
- OWEN, J. Prostaglandin  $E_2$  and the bovine sphincter pupillae. *Brit. J. Pharmacol.* 1973. 49 415-427.
- ALMER, J. H., Antagonism of prostaglandin  $E_2$  by 1-acetyl-2-( $\beta$ -chloro-10,11-dihydrochene(b,f) (1,4)-enoxypae-10-carboxyl) hydrazine (SC 19230). *Arch. int. Pharmacodyn.* 1969 180 46-56.
- CHAIKIN, U. and W. P. KOELLA, Release of isolated pig iris sphincter to electrical stimulation and acetylcholine. *Amer. J. Physiol.* 1964. 206. 255-261.
- CHAIKIN, U. and W. P. KOELLA, Adrenergic innervation of cat iris sphincter. *Amer. J. Physiol.* 1964 b. 207 175-178.
- DAW, J. E. and P. W. RAINWELL, Inhibition of gastric secretion in rats by prostaglandin  $E_1$ . In *Prostaglandins* 5<sup>th</sup> ed. of the *Werner Forum for Experimental Biology* Eds. P. W. Rainwell and J. E. Shaw. Interscience, N. Y. 1968. pp. 55-64.
- NATZMAN, M. B. and C. D. KUO, Prostaglandin influences on intracocular pressure and pupil size. *Amer. J. Physiol.* 1967 212. 329-334.
- BERNHALM, A. and P. HEDQVIST, Inhibition by prostaglandin  $E_1$  of parasympathetic neurotransmission in the rabbit heart. *Life Sci* 1971. 30. 445-470.

Therefore, the potentiating effect of the PGs must be regarded an interaction with cholinergic neuroeffector transmission.

It should be noted that, although the PGs were tested over a wide range of doses, inhibition of the contraction response was never seen. This is interesting from the point of view that PGs have been reported to inhibit effector responses to vagal nerve stimulation (Shaw and Ramwell 1968, Wennmalm and Hedqvist 1971), and indicates that the effect of PGs on cholinergically innervated tissues is not unitarily stimulant or inhibitory.

The fact that the responses to acetylcholine were enhanced by the PGs indicated that the PGs exert at least part of their stimulant effect on neuroeffector transmission in the sphincter muscle postfunctionally. However, an additional prefunctional effect on acetylcholine release cannot be ruled out as has also been suggested for the stimulant action of  $\text{PGF}_{2\alpha}$  on saliva secretion from the canine submandibular gland (Hahn and Patil 1974). Further studies on transmitter release from the nerve endings in the sphincter muscle are required to establish this possibility.

The presence of PG in, and release from, the iris sphincter muscle has been documented (Ånggård and Samuelsson 1964, Posner 1973). Moreover, increased PG release seems to occur during periods of increased tone (Posner 1973), and a high tone can be effectively counteracted by PG synthesis inhibitors (Gustafsson *et al.* 1973) or by PG effector blockade (Posner 1973, present results). These observations merit the assumption that locally formed PG modulates or even generates the intrinsic tone of the iris sphincter muscle as has been postulated for the smooth muscle of the gut (Ferreira *et al.* 1972). However, inhibition of neurogenically elicited contractions by PG effector blockade and enhancement by exogenous PG in concentrations below those which increase the tone of the muscle invites the intriguing possibility that endogenous PG, perhaps to an even greater extent, serves the function of controlling neuroeffector transmission in the sphincter muscle.

This investigation was supported by grants from Stiftelsen Lars Hierta Minne and from The Swedish Medical Research Council project no 04X-434.

## References

- AMBACHE, N. and H. C. BRUNNER, A simple chemical procedure for distinguishing E from F prostaglandins with application to tissue extracts. *Brit. J. Pharmacol.* 1968 33 16-170.
- AMBACHE, N., H. C. BRUNNER, J. G. ROSS and J. WHITING, Thin-layer chromatography of spermogenic unsaturated hydroxyacids from various tissues. *J. Physiol. (Lond.)* 1966, 123 77P-78P.
- AMBACHE, N., L. KAVANAUGH and J. WHITING, Effect of mechanical stimulation on rabbit eyes: release of active substance in anterior chamber perfusates. *J. Pharmacol. (Lond.)* 1965, 176 378-408.
- ÅNGGÅRD, E. and B. SAMUELSSON, Smooth-muscle stimulating lipids in sheep iris. The identification of prostaglandin  $\text{F}_{2\alpha}$ . *Biochem. Pharmacol.* 1964 13 281-283.
- BEITCH, B. R. and K. E. EAKIN, The effects of prostaglandins on the intraocular pressure of the rabbit. *Brit. J. Pharmacol.* 1969 37 158-167.
- BERGSTROM, S., R. ELIASSON, U. S. v. EULER and J. SJÖVALL, Some biological effects of 1-crySTALLINE prostaglandin factors. *Acta physiol. scand.* 1959 43 133-144.
- BOURA, A. L. A. and F. A. GREEN, Adrenergic neurooc blocking agents. *Ann. Rev. Pharmacol.* 1965, 5, 183-191.
- DORF, D. A. van, G. H. JOUVENAZ and C. B. STRUICK, The biosynthesis of prostaglandin in pig eye iris. *Biochim. biophys. Acta (Amst.)* 1967 137 396.

- FERRIERA, S. H. A. HERMAN and J. R. VANE, Prostaglandin generation maintains the smooth muscle tone of the rabbit isolated jejunum. *Brit. J. Pharmacol.* 1972. 44 328-330.
- OSTERLIND, L. E., P. HEDQVIST and H. LAOENCRANTZ, Prostaglandin mediated enhancement of effector response to cholinergic nerve stimulation. *Acta physiol. scand.* 1973. Suppl. 396. 104.
- HAIN, R. A. and P. N. P. TIL, Salivation induced by prostaglandin  $P_{GE}$  and modification of the response by atropine and physostigmine. *Brit. J. Pharmacol.* 1972. 44 527-533.
- HAIN, R. A. and P. N. P. TIL, Further observations on the interaction of  $PGF_{2\alpha}$  with cholinergic mechanisms in canine salivary glands. *Europ. J. Pharmacol.* 1974. 25 279-286.
- HEDQVIST, P. Studies of the effect of prostaglandins  $E_1$  and  $E_2$  on the sympathetic neurovascular transmission in some animal tissues. *Acta physiol. scand.* 1970. 79 Suppl. 345 1-40.
- HEDQVIST, P. Prostaglandin mediated control of sympathetic neuroeffector transmission. *Adv. Biosci.* 1972. 9 461-472.
- HORTON, E. W. Biological activities of pure prostaglandins. *Experientia (Basel)* 1965. 21 113-118.
- P. TIL, P. H. Adrenergic receptors of the bovine iris sphincter. *J. Pharmacol. exp. Ther.* 1969. 166. 299-307.
- POWELL, J. The release of prostaglandin  $E_2$  from the bovine iris. *Brit. J. Pharmacol.* 1970. 40. 163P 164P.
- POWELL, J. The action of prostaglandins on the bovine sphincter pupillae. *J. Physiol. (Lond.)* 1971. 227 25P 26P.
- POWELL, J. Prostaglandins  $E_2$  and the bovine sphincter pupillae. *Brit. J. Pharmacol.* 1972. 49 415-427.
- SANDEL, J. H. Antagonism of prostaglandin  $E_2$  by 1-acetyl-3-(8-chloro-10,11-dihydrobenzo(b,f)(1,4)-oxazepine-10-carboxyl) hydrazine (JC 19220). *Arch. int. Pharmacodyn.* 1969. 180. 44-56.
- SCHAEFFEL, U. and W. P. KOELLA, Reaction of isolated pig iris sphincter to electrical stimulation and acetylcholine. *Amer. J. Physiol.* 1964. 205 255-261.
- SCHAEFFEL, U. and W. P. KOELLA, Adrenergic innervation of cat iris sphincter. *Amer. J. Physiol.* 1964 b. 207 273-278.
- SHAW, J. E. and P. W. RASTWELL, Inhibition of gastric secretion in rats by prostaglandin  $E_2$ . In *Prostaglandins Symp. of the Worcester Found. for Experimental Biology* Eds. P. W. Rastweli and J. E. Shaw (Interscience, N. Y. 1966. pp. 55-64).
- WATZMAN, M. B. and C. D. KING, Prostaglandin influence on intracellular pressure and pupil size. *Amer. J. Physiol.* 1967. 212. 329-334.
- WERNBERG, Å. and P. HEDQVIST, Inhibition by prostaglandin  $E_1$  of parasympathetic neurotransmission in the rabbit heart. *Life Sci.* 1971. 10. 445-470.

Therefore, the potentiating effect of the PGs must be regarded an interaction with cholinergic neuroeffector transmission.

It should be noted that, although the PGs were tested over a wide range of doses, inhibition of the contraction response was never seen. This is interesting from the point of view that PGs have been reported to inhibit effector responses to vagal nerve stimulation (Shaw and Ramwell 1968, Wennmalm and Hedqvist 1971), and indicates that the effect of PGs on cholinergically innervated tissues is not unitarily stimulant or inhibitory.

The fact that the responses to acetylcholine were enhanced by the PGs indicated that the PGs exert at least part of their stimulant effect on neuroeffector transmission in the sphincter muscle postjunctionally. However an additional prejunctional effect on acetylcholine release cannot be ruled out as has also been suggested for the stimulant action of  $\text{PGF}_{2\alpha}$  on saliva secretion from the canine submandibular gland (Hahn and Pail 1974). Further studies on transmitter release from the nerve endings in the sphincter muscle are required to establish this possibility.

The presence of PG in, and release from, the iris sphincter muscle has been documented (Ånggård and Samuelsson 1964, Posner 1973). Moreover increased PG release seems to occur during periods of increased tone (Posner 1973), and a high tone can be effectively counteracted by PG synthesis inhibitors (Gustafsson *et al* 1973) or by PG effector blockade (Posner 1973, present results). These observations merit the assumption that locally formed PG modulates or even generates the intrinsic tone of the iris sphincter muscle as has been postulated for the smooth muscle of the gut (Ferreira *et al* 1972). However inhibition of neurogenically elicited contractions by PG effector blockade and enhancement by exogenous PG in concentrations below those which increase the tone of the muscle invites the intriguing possibility that endogenous PG perhaps to an even greater extent serves the function of controlling neuroeffector transmission in the sphincter muscle.

This investigation was supported by grants from Stiftelsen Lars Hiertas Minne and from The Swedish Medical Research Council project no 04X-4342.

## References

- AMBLACHE, N. and H. C. BRUMMER, A simple chemical procedure for distinguishing E from F prostaglandins, with application in tissue extracts. *Brit. J. Pharmacol.* 1968 33 162-170.
- AMBLACHE, N., H. C. BRUMMER, J. G. ROSE and J. WHITTING, Thin-layer chromatography of stereoisomeric unsaturated hydroxyacids from various tissues. *J. Physiol. (Lond.)* 1966, 183 77P-78P.
- AMBLACHE, N., L. KAVANAGH and J. WHITTING, Effect of mechanical stimulation on rabbits' eyes: release of active substance in anterior chamber perfusates. *J. Physiol. (Lond.)* 1965 176 378-408.
- ÅNGGÅRD E. and B. SAMUELSSON, Smooth-muscle stimulating lipids in sheep iris. The identification of prostaglandin  $\text{F}_{2\alpha}$ . *Biochem. Pharmacol.* 1964 13 281-283.
- BEITCH, H. R. and K. E. EAKINS, The effects of prostaglandins on the intraocular pressure of the rabbit. *Brit. J. Pharmacol.* 1969 37 158-167.
- BERGSTRÖM, S., R. ELIASSON, U. S. V. ELMER and J. SÖFVALL, Some biological effects of 1-crySTALLINE prostaglandin factors. *Acta physiol. scand.* 1959 45 133-144.
- BOURA, A. L. A. and F. A. GREEN, Adrenergic neuroeffector blocking: general. *Ann. Rev. Pharmacol.* 1965, 5 183-212.
- DORF, D. A. van, G. H. JOUVENAZ and C. B. STRUICK, The biosynthesis of prostaglandin in pig eye iris. *Biochim. biophys. Acta (Amst.)* 1967 137 396.

Pulmonary flow and pressure, as well as pulmonary blood volume and extravascular lung water content were measured. Although large effects on vascular pressures were seen, only small changes were detected as regards fluid contents in the lung.

A preliminary report on some of the results has been given elsewhere (Karlsen, Aarseth and Bo 1973).

### Methods

Canine weighing 2.3-5.2 kg were used. Anaesthesia was induced by ether inhalation. A catheter was then inserted into femoral vein and chloralose (30 mg/kg b.w.) was given i.v. The animals were tracheostomized, cannulated (Altoferm, Roche, 0.5 mg/kg b.w.), and given positive pressure ventilation by an Ideal Searling pump. The pump frequency was 33 strokes/min, and end expiratory pressure was kept at 2 cm H<sub>2</sub>O. The tidal volume was regulated so as to keep pH of arterial blood between 7.35 and 7.45. At 30 min intervals "deep breath" was given by briefly increasing the inflation pressure to 18-20 cm H<sub>2</sub>O.

A cannula was inserted into the thorax. Catheters were inserted into the pulmonary artery through the wall of the right ventricle and into the left atrium through its appendage. Catheters were also inserted into both femoral arteries. Intravascular pressures were recorded by Statham pressure transducers (P23Db and P23Dc) connected to Grass multichannel recorder Model 7B. An electromagnetic flowprobe mounted on the ascending aorta was connected to flowmeter (Nycotron, Norway), and intratracheal as well as aortic flow were continuously followed on the Grass recorder.

Eight arteries were loosely placed around the roots of the two upper and the right middle lung lobes. These wires were later tightened one by one. Thereby the circulation through one particular lobe was arrested and the lobe could be removed for evaluation of its water and blood content. As these lobe parameters are calculated relative to the wet dry weight of the lobes, the values obtained for the 3 lobes could be compared. All lobe-isolations were performed at the end of an expiration and done with transpulmonary pressure of 2 cm H<sub>2</sub>O.

When the usual surgical procedures had been finished, the animals were left undisturbed for 45 min. A blood had sample been withdrawn, and the erythrocytes labelled with <sup>51</sup>Cr and washed thoroughly (Aarseth 1970). New these erythrocytes were mixed with an equal amount of <sup>51</sup>Cr-labelled known serum fraction. A weighed sample of this mixture was injected to the animal, and 10 min later the artery as situated around the root of the right upper lobe simultaneously arterial blood was sampled for haematocrit measurement and for analysis of its isotope content.

Up to this point of the procedure all animals were given the same handling. The second and third lobes, to be removed later, should however reflect changes brought about by catecholamines or hypoxic stimulation. Therefore one of these types of stimulation was initiated 8 min before the second lobectomy and maintained continuously for 25 min until the third lobe was removed. Thus the effects of catecholamine-stimulation as well as of general hypoxia were studied after 8 as well as after 25 min of stimulus administration.

The following groups were established.

Control group: 5 animals where the 3 lobes were removed without any intervening procedure.

Adrenaline group: 7 animals receiving 5 µg/kg b.w./min of adrenaline.

Noradrenaline group: (a) 4 animals receiving 5 µg/kg b.w./min of noradrenaline and (b) 4 other animals receiving 1.25 µg/kg b.w./min of noradrenaline.

Hypoxic group: 4 animals are ventilated with gas mixture of air and nitrogen with P<sub>O<sub>2</sub></sub> of about 75 mm Hg.

P<sub>O<sub>2</sub></sub> was measured in arterial blood samples from all animals throughout the experiments. For this Radiometer oxygen electrode was used.

**Lung blood and extravascular water content.** The removed lobes were frozen in liquid nitrogen and thereafter cut so that the tissue could be divided into several test tubes. These tissue samples and also the blood samples taken were analyzed for their isotope content. Their wet weight were found, and they were then dried at 75°C until the weight became stable. From the radioactivity in blood and tissue, and from the wet and dry weights of the samples, the blood content and extravascular water content in the lobes are calculated relative to the wet dry tissue weight of that lobe. These weight-related volumes could then be compared from lobe to lobe. In each animal the first lobe gave the reference values for that animal. The values for blood and water content in the second and third lobes were then expressed as per cent of these reference values. A more detailed description of the calculations has been given previously (Aarseth and Bo 1972). Statistical calculations have been done by two-sided Wilcoxon's two-sample test.

## Effects of Catecholamine-Infusions and Hypoxia on Pulmonary Blood Volume and Extravascular Lung Water Content in Cats

By

P. AARSETH, J. KARLSEN and G. BØ

Received 14 February 1975

---

### Abstract

AARSETH, P., J. KARLSEN and G. BØ: *Effects of catecholamine-infusions and hypoxia on pulmonary blood volume and extravascular lung water content in cats* Acta physiol. scand. 1975 95 34-40

The effects of catecholamine-infusions and of hypoxia on lung blood volume and on extravascular lung water content have been studied in anesthetized cats with opened chests. To this end biopsy technique, with isotope labelling of blood and with successive removal of the smaller lung lobes, was employed. Mean pulmonary arterial pressure increased by 30-75% upon infusing catecholamines or upon inducing general hypoxia, the latter stimulus being the more powerful one. Pulmonary blood volume did not increase during these procedures, where a active constriction of pulmonary vessels thus apparently took place. The extravascular lung water content was found to be reduced in animals infused with norepinephrine or ventilated by hypoxic air, whereas a small increase was observed in the animals receiving adrenaline. The difference might reflect domination of precapillary vasoconstriction in the former groups, whereas post capillary vasoconstriction could be more pronounced with adrenal ad.

---

It has been suggested that increased lung water content with impairment of lung function develops upon elevation of the blood level of catecholamines and upon increased sympathetic nervous activity. Such adrenergic effects are thought to be involved in the pulmonary edema induced by increased intracranial pressure (Bean and Beckman 1969) and a variety of neurogenic stimuli (Luisada 1967). Accidental injections of large amounts of adrenaline to humans can also give rise to an acute, fulminant pulmonary edema (Ersöz and Finestock 1971).

A wet lung syndrome seen as a consequence of traumatic and hypovolemic shock is also characterized by congestion and interstitial edema (Moore *et al.* 1969). In experimental studies pulmonary effects of traumatic and hemorrhagic shock seemed to be prevented when hormonal release from the adrenal medulla was eliminated (Bø and Hognestad 1971).

In the present work we have attempted to evaluate the effects on the pulmonary vasculature of infused catecholamines, with an infusion rate comparable to maximal endogenous hormonal release. In addition one group of animals was tested with general hypoxemia, which is again known to be a powerful stimulus for endogenous release of catecholamines.

Percentage change after administration for  
8 min  $\square$  or 28 min  $\blacksquare$

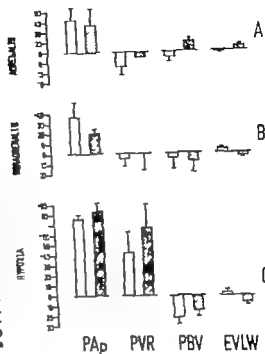


Fig. 1. Development of various pulmonary parameters upon catecholamine infusions or generalized hypoxemia. In cat lungs *in situ*, the arterial pressure ( $PA_p$ ), the vascular resistance (PVR), the blood volume (PBV) and the extravascular water content (EVLW) were measured after either catecholamine infusion or ventilation hypoxemia for 8 min and for 28 min respectively. The values given represent the percentage changes from control values obtained in each animal before stabilization. A) adrenaline, 5  $\mu\text{g/kg}$  b.w./min; (B) noradrenaline, 1.25 or 5  $\mu\text{g/kg}$  b.w./min; (C) hypoxemia, arterial  $P_{O_2}$  = 24–29 mm Hg. Mean values  $\pm$  S.E.

left atrial pressure increased during the adrenaline infusion, but the mean value after 28 min was only 8 mm Hg, and in only one animal did it exceed 10 mm Hg.

**Effects of noradrenaline (Fig. 1 B).** There seemed to be no differences on the effects caused by noradrenaline given at the two rates selected, 1.25  $\mu\text{g/kg}$  b.w./min and 5  $\mu\text{g/kg}$  b.w./min.

All animals given noradrenaline were therefore grouped together. Upon these infusions cardiac output and the pulmonary arterial pressure increased to the same extent by some 25%. The pulmonary vascular resistance was thus unchanged during infusion of noradrenaline. The changes in lung blood content were small and inconsistent, whereas the extravascular lung water content showed a small decrease on the border of being significant ( $p = 0.07$ ). After 28 min of noradrenaline-infusion there was also a tendency towards increased left atrial pressure, but the changes were small and not statistically significant.

**Effects of general hypoxemia (Fig. 1 C).** The ventilating gas for the 4 animals in this group had an oxygen tension of about 75 mm Hg. The resulting arterial  $P_{O_2}$  was measured to be 24–29 mm Hg. Despite this grave hypoxemia the cardiac output increased and remained high throughout the experimental period. The pulmonary arterial pressure increased, however much more markedly (by nearly 100%) than did the cardiac output, indicating an increased pulmonary vascular resistance. Despite the tremendous increase in lung arterial pressure, the pulmonary blood content, when looking at the 2 removed lobes together, was



TABLE I The effects of lobectomies on circulatory parameters in remaining lung in cats. Three small lobes were removed one by one. The values for pulmonary vascular resistance, pulmonary artery pressure and cardiac output are the ones found immediately before the removal of the different lobes. Lung blood content and lung extravascular water are the values found in each lobe, calculated relative to the wet dry tissue weight of that lobe. For the first lobe the absolute values are given, while for the second and third lobe the percentage change from the first lobe are given. Mean values  $\pm$  S.D.

	Lobe No. 1—100%	Lobe No. 2 %	Lobe No. 3 %
Pulmonary vascular resistance	$0.039 \pm 0.04$ mm Hg $\text{ml}^{-1} \times \text{min}$	$118 \pm 9$	$141 \pm 9$
Pulmonary arterial pressure	$15.4 \pm 4.3$ mm Hg	$102 \pm 5$	$111 \pm 3$
Cardiac output	$304 \pm 100$ ml/min	$88 \pm 3$	$81 \pm 7$
Lung blood content	$4.4 \pm 1.4$ ml/g dry tissue weight	$114 \pm 14$	$100 \pm 6$
Lung extravascular water	$4.69 \pm 0.09$ ml/g dry tissue weight	$95 \pm 2$	$100 \pm 1$

### Results

The present method used for determination of lung fluid contents involved three successive lobectomies. These lobectomies might *per se* influence the parameters studied. Such influences could be reflected in the control group of 5 animals and the data from this group are presented in Table I. As the pulmonary vascular bed was reduced by the lobectomies, the lung vascular resistance increased from  $0.039$  mm Hg  $\text{ml}^{-1} \text{min}$  initially to  $0.052$  mm Hg  $\text{ml}^{-1} \text{min}$ , when the third lobe was removed. The cardiac output decreased some 10–20% during the experimental period, while the mean pulmonary arterial pressure increased from 15 mm Hg to 17 mm Hg at the time of the third lobectomy. The blood content increased by 14 and 22% respectively in the second and third lobe, while the lung extravascular water content decreased by some 5% in the second lobe and returned towards the initial level in the third lobe.

When in the following we attempted to evaluate the effects of catecholamines and hypoxemia proper, the above mentioned changes induced by the lobectomies *per se* were subtracted from the changes found in the various test groups of animals. In Fig. 1 A–C are shown the net effects thus obtained.

All animals (except the ones given hypoxic air) had a normal arterial  $P_{O_2}$  level at the beginning of the experiment (mean value 98 mm Hg). All animals (except the ones given hypoxic air) also had an arterial blood  $P_{O_2}$  above 65 mm Hg (mean value 88 mm Hg) at the end of the experiment.

*Effects of adrenaline (Fig. 1 A)* Aortic flow increased by about 40% when adrenaline was infused at a constant rate of  $5 \mu\text{g/kg b.w./min}$ . Even though this marked increase in cardiac output was not maintained throughout the 28 min of infusion, there was still an augmented cardiac flow at the end of the experiment ( $p=0.05$ ). The pulmonary arterial pressure increased by about 25% ( $p=0.01$ ), but the pulmonary vascular resistance seemed to decrease as compared to the value seen in the control group. The changes in blood content of the lung lobes were small and inconsistent. The extravascular water content of the lobes was found to be unchanged after 8 min of infusion, but there may have been a small increase in the third lobes when these were compared to those of the control group ( $p=0.15$ ). The

Percentage change after administration for  
0 min. □ or 28 min. ▤

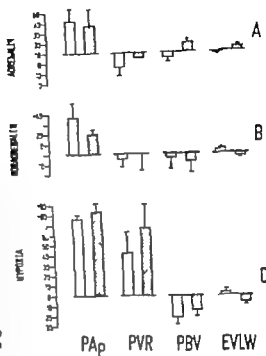


Fig. 1. Development of various pulmonary parameters upon catecholamine infusion or generalized hypoxemia. In cat lungs in situ, the arterial pressure ( $PA_p$ ), the vascular resistance (PVR), the blood volume (PBV) and the extravascular water content (EVLW) were estimated after either catecholamine infusion or ventilation hypoxia for 8 min and for 28 min respectively. The values given represent the percentage changes from control values obtained in each animal before stimulation with: (A) adrenaline, 5  $\mu\text{g/kg}$  b.w./min, (B) noradrenaline, 1.25 or 5  $\mu\text{g/kg}$  b.w./min, (C) tyrosine, arterial  $P_{O_2}$  24–29 mm Hg. Mean  $\pm$  S.E.

left atrial pressure increased during the adrenaline infusion, but the mean value after 28 min was only 8 mm Hg, and in only one animal did it exceed 10 mm Hg.

**Effects of noradrenaline (Fig. 1 B).** There seemed to be no differences on the effects caused by noradrenaline given at the two rates selected, 1.25  $\mu\text{g/kg}$  b.w./min and 5  $\mu\text{g/kg}$  b.w./min.

All animals given noradrenaline were therefore grouped together. Upon these infusions, cardiac output and the pulmonary arterial pressure increased to the same extent by some 23%. The pulmonary vascular resistance was thus unchanged during infusion of noradrenaline. The changes in lung blood content were small and inconsistent, whereas the extravascular lung water content showed a small decrease on the border of being significant ( $p = 0.07$ ). After 28 min of noradrenaline-infusion there was also a tendency towards increased left atrial pressure, but the changes were small and not statistically significant.

**Effects of general hypoxemia (Fig. 1 C).** The ventilating gas for the 4 animals in this group had an oxygen tension of about 75 mm Hg. The resulting arterial  $P_{O_2}$  was measured to be 24–29 mm Hg. Despite this grave hypoxemia the cardiac output increased and remained high throughout the experimental period. The pulmonary arterial pressure increased, however, much more markedly (by nearly 100%) than did the cardiac output, indicating an increased pulmonary vascular resistance. Despite the tremendous increase in lung arterial pressure, the pulmonary blood content, when looking at the 2 removed lobes together, was

TABLE 1 The effects of lobectomies on circulatory parameters in remaining lung in cats. Three small lobes were removed one by one. The values for pulmonary vascular resistance, pulmonary artery pressure and cardiac output are the ones found immediately before the removal of the different lobes. Lung blood content and lung extravascular water are the values found in each lobe & calculated relative to the net dry tissue weight of that lobe. For the first lobe the absolute value are given, while for the second and third lobe the percentage change from the first lobe are given. Mean values  $\pm$  S.D.

	Lobe No. 1 = 100 %	Lobe No. 2 %	Lobe No. 3 %
Pulmonary vascular resistance	$0.039 \pm 0.04$ mm Hg $\text{ml}^{-1} \times \text{min}$	$118 \pm 9$	$141 \pm 9$
Pulmonary arterial pressure	$15.4 \pm 4.3$ mm Hg	$10. \pm 5$	$111 \pm 3$
Cardiac output	$304 \pm 100$ ml/min	$88 \pm 3$	$81 \pm 7$
Lung blood content	$4.4 \pm 1.4$ ml/g dry tissue weight	$114 \pm 14$	$122 \pm 6$
Lung extravascular water	$4.69 \pm 0.09$ ml/g dry tissue weight	$95 \pm 2$	$100 \pm 1$

### Results

The present method used for determination of lung fluid contents involved three successive lobectomies. These lobectomies might *per se* influence the parameters studied. Such influences could be reflected in the control group of 5 animals and the data from this group are presented in Table I. As the pulmonary vascular bed was reduced by the lobectomies, the lung vascular resistance increased from  $0.039 \text{ mm Hg} \times \text{ml}^{-1} \times \text{min}$  initially to  $0.052 \text{ mm Hg} \times \text{ml}^{-1} \times \text{min}$ , when the third lobe was removed. The cardiac output decreased some 10–20% during the experimental period, while the mean pulmonary arterial pressure increased from 15 mm Hg to 17 mm Hg at the time of the third lobectomy. The blood content increased by 14 and 22% respectively in the second and third lobe, while the lung extravascular water content decreased by some 5% in the second lobe and returned towards the initial level in the third lobe.

When in the following we attempted to evaluate the effects of catecholamines and hypoxemia proper the above mentioned changes induced by the lobectomies *per se* were subtracted from the changes found in the various test groups of animals. In Fig. 1 A–C are shown the *net* effects thus obtained.

All animals (except the ones given hypoxic air) had a normal arterial  $P_{O_2}$  level at the beginning of the experiment (mean value 98 mm Hg). All animals (except the ones given hypoxic air) also had an arterial blood  $P_{O_2}$  above 65 mm Hg (mean value 88 mm Hg) at the end of the experiment.

*Effects of adrenaline (Fig. 1 A).* Aortic flow increased by about 40% when adrenaline was infused at a constant rate of  $5 \mu\text{g/kg b.w.}/\text{min}$ . Even though this marked increase in cardiac output was not maintained throughout the 28 min of infusion there was still an augmented cardiac flow at the end of the experiment ( $p < 0.05$ ). The pulmonary arterial pressure increased by about 25% ( $p < 0.01$ ) but the pulmonary vascular resistance seemed to decrease as compared to the value seen in the control group. The changes in blood content of the lung lobes were small and inconsistent. The extravascular water content of the lobes was found to be unchanged after 8 min of infusion but there may have been a small increase in the third lobes when these were compared to those of the control group (

Percentage change after administration for  
8 min  $\square$  or 28 min  $\square$

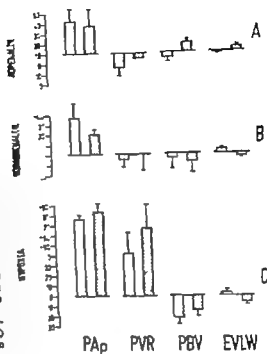


Fig. 1. Development of various pulmonary parameters upon catecholamine infusions or generalized hypoxia. In our lungs in situ, the arterial pressure (P<sub>Ap</sub>), the vascular resistance (P<sub>VR</sub>), the blood volume (P<sub>BV</sub>) and the extravascular water content (EVLW) were estimated after either catecholamine infusion or ventilation hypoxia for 8 min and for 28 min respectively. The values given represent the percentage changes from control values obtained in each animal before anaesthesia (A) adrenaline, 5  $\mu\text{g/kg}$  b.w./min, (B) noradrenaline, 1.25 or 5  $\mu\text{g/kg}$  b.w./min (C) hypoxia, arterial P<sub>O<sub>2</sub></sub> 24–29 mm Hg. Mean values  $\pm$  S.E.

left atrial pressure increased during the adrenaline infusion, but the mean value after 28 min was only 8 mm Hg, and in only one animal did it exceed 10 mm Hg.

**Effects of noradrenaline (Fig. 1 B).** There seemed to be no differences on the effects caused by noradrenaline given at the two rates selected, 1.25  $\mu\text{g/kg}$  b.w./min and 5  $\mu\text{g/kg}$  b.w./min. All animals given noradrenaline were therefore grouped together. Upon these infusions cardiac output and the pulmonary arterial pressure increased to the same extent by some 25%. The pulmonary vascular resistance was thus unchanged during infusion of noradrenaline. The changes in lung blood content were small and inconsistent, whereas the extravascular lung water content showed a small decrease on the border of being significant ( $p = 0.07$ ). After 28 min of noradrenaline-infusion there was also a tendency towards increased left atrial pressure, but the changes were small and not statistically significant.

**Effects of general hypoxemia (Fig. 1 C).** The ventilating gas for the 4 animals in this group had an oxygen tension of about 75 mm Hg. The resulting arterial P<sub>O<sub>2</sub></sub> was measured to be 24–29 mm Hg. Despite this grave hypoxemia the cardiac output increased and remained high throughout the experimental period. The pulmonary arterial pressure increased, however much more markedly (by nearly 100%) than did the cardiac output, indicating an increased pulmonary vascular resistance. Despite the tremendous increase in lung arterial pressure the pulmonary blood content, when looking at the 2 removed lobes together was

TABLE I The effects of lobectomies on circulatory parameters in remaining lung in cats. Three small lobes were removed one by one. The values for pulmonary vascular resistance, pulmonary arterial pressure and cardiac output are the ones found immediately before the removal of the different lobes. Lung blood content and lung extravascular water are the values found in each lobe calculated relative to the net dry tissue weight of that lobe. For the first lobe the absolute values are given, while for the second and third lobe the percentage change from the first lobe are given. Mean values  $\pm$  S.D.

	Lobe No. 1—100%	Lobe No. 2 %	Lobe No. 3 %
Pulmonary vascular resistance	$0.039 \pm 0.04$ mm Hg $\times$ ml <sup>-1</sup> min	$118 \pm 9$	$141 \pm 9$
Pulmonary arterial pressure	$15.4 \pm 4.3$ mm Hg	$102 \pm 5$	$111 \pm 3$
Cardiac output	$304 \pm 100$ ml/min	$88 \pm 3$	$81 \pm 7$
Lung blood content	$4.4 \pm 1.4$ ml/g dry tissue weight	$114 \pm 14$	$122 \pm 6$
Lung extravascular water	$4.69 \pm 0.09$ ml/g dry tissue weight	$95 \pm 2$	$100 \pm 1$

### Results

The present method used for determination of lung fluid contents involved three successive lobectomies. These lobectomies might *per se* influence the parameters studied. Such influences could be reflected in the control group of 5 animals and the data from this group are presented in Table I. As the pulmonary vascular bed was reduced by the lobectomies, the lung vascular resistance increased from  $0.039$  mm Hg  $\times$  ml<sup>-1</sup> min initially to  $0.052$  mm Hg  $\times$  ml<sup>-1</sup> min when the third lobe was removed. The cardiac output decreased some 10–20% during the experimental period while the mean pulmonary arterial pressure increased from 15 mm Hg to 17 mm Hg at the time of the third lobectomy. The blood content increased by 14 and 22% respectively in the second and third lobe, while the lung extravascular water content decreased by some 5% in the second lobe and returned towards the initial level in the third lobe.

When in the following we attempted to evaluate the effects of catecholamines and hypoxemia proper, the above mentioned changes induced by the lobectomies *per se* were subtracted from the changes found in the various test groups of animals. In Fig. 1 A–C are shown the *net* effects thus obtained.

All animals (except the ones given hypoxic air) had a normal arterial  $P_{O_2}$  level at the beginning of the experiment (mean value 98 mm Hg). All animals (except the ones given hypoxic air) also had an arterial blood  $P_{O_2}$  above 65 mm Hg (mean value 88 mm Hg) at the end of the experiment.

*Effects of adrenaline (Fig. 1 A).* Aortic flow increased by about 40% when adrenaline was infused at a constant rate of  $5 \mu\text{g/kg b.w./min}$ . Even though this marked increase in cardiac output was not maintained throughout the 28 min of infusion, there was still an augmented cardiac flow at the end of the experiment ( $p < 0.05$ ). The pulmonary arterial pressure increased by about 25% ( $p < 0.01$ ) but the pulmonary vascular resistance seemed to decrease as compared to the value seen in the control group. The changes in blood content of the lung lobes were small and inconsistent. The extravascular water content of the lobes was found to be unchanged after 8 min of infusion, but there may have been a small increase in the third lobes when these were compared to those of the control group ( $p < 0.05$ ). The

1973) as well as in cat lungs *in situ* (Aarseth, Be and Hauge 1975). The latter of these works demonstrated that a substantial increase in left atrial pressure (and thus also in pulmonary capillary pressure) was needed in order to increase lung water content some few per cent. Thus left atrial pressure had to be elevated to about 10 mm Hg in order to achieve a 3% increase in water content (Aarseth, Be and Hauge 1975). Two thirds of such a pressure increase will probably extend to the pulmonary capillaries. In the present experiments with infusion of adrenaline, the pulmonary arterial pressure increased from about 12 to 18 mm Hg, and the left atrial pressure increased from about 5 to about 8 mm Hg during the infusion. If there is predominance of postcapillary constriction (reduced distensibility) and at the same time some precapillary vasodilatation (reduced resistance), these pressure changes might result in a capillary pressure increase of as much as 7 mm Hg. The findings of a 4% increase in extravascular lung water content would then be in good agreement with the above mentioned results of Aarseth, Be and Hauge (1975).

The small reduction in lung extravascular water seen after noradrenaline infusion for 28 min, appears to indicate a predominant precapillary action of this drug. The lung arterial pressure increase was thus similar to that seen after infusion of adrenaline but the vascular resistance was not apparently reduced. Also, the pulmonary blood volume seemed to be smaller after noradrenaline infusion than after adrenaline, again indicating a more marked upstream location of the noradrenaline induced vasoconstriction.

The infusion rate of adrenaline used in the present experiments was 5  $\mu\text{g/kg}$  b.w./min, which is about equal to the maximal release rate from the cat's adrenal medulla (Celander 1954). For the cat it seems reasonable to conclude therefore that physiological doses of adrenaline can give substantial increases in pulmonary capillary pressure, without any pooling of blood in the lungs. Left ventricular failure has not been involved as there was no or only small increases in left atrial pressure.

Hypoxia was included in the present experiments because hypoxemia was regarded to be very potent stimulus for generalized sympathetic discharge and for adrenal catecholamine release. Hypoxemia and increased sympathetic activity appear to be essential events in many critically ill and injured patients suffering from the so-called wet lung syndrome. At the same time ventilation hypoxia will have a direct vasomotor effect on the lungs. Alveolar hypoxia will thus cause pulmonary constriction and hypertension through a specific mechanism involving histamine (Hauge and Stamb 1969). Wayne and Severinghouse (1968) have shown that hypoxia-induced pulmonary vasoconstriction is located in precapillary vessels.

The resulting effect on the pulmonary vasculature of ventilation hypoxia on the one hand and of an increased sympathetic discharge rate and of increased adrenal catecholamine release on the other cannot easily be predicted.

In the present experiments the  $P_{aO_2}$  was kept as low as 11 mm Hg for half an hour but this very grave hypoxemia was well tolerated. The cardiac output was slightly increased throughout the experiment, and the femoral arterial pressure showed only a very moderate reduction (to about 100 mm Hg) at the end of the experiment. The left atrial pressure increased by 2.3 mm Hg. Left cardiac failure was thus apparently not evolving. The hypoxemia gave an increase in pulmonary arterial pressure by some 75% (Fig. 1 C). Both the blood volume and the extravascular water content of the lungs were, if anything, reduced after half an

found to be reduced by about 25% during hypoxemia ( $p=0.001$ ). The extravascular lung water content changed in the same direction as was the case during infusion of noradrenaline. Thus the water content in the lung after 28 min with hypoxia seemed to be slightly reduced as compared to the situation in the control animals ( $p=0.055$ ). The left atrial pressure increased by a mean value of only 2–3 mm Hg during the hypoxic period.

### Discussion

When catecholamines are given to the intact animals, the pulmonary vascular changes seen will reflect both direct effects on the lung vessels, as well as influences on these vessels secondary to effects in the systemic circulation or on the right heart. In isolated, perfused cat and rabbit lungs Hauge, Lunde and Waaler (1967) have demonstrated that both adrenaline and noradrenaline reduce (through an  $\alpha$ -receptor mechanism) pulmonary vascular volume. The two drugs differed in their effects on the resistance vessels, as adrenaline caused distention ( $\beta$ -receptors) and noradrenaline predominantly constriction ( $\alpha$ -receptors). When catecholamines are given to the intact animal, results have been achieved which suggest that vasoconstriction of blood vessels in the systemic circulation can cause translocation of blood to the distensible low-pressure vessels of the lesser circulation (Luisada 1967).

In the present experiments, however the direct effects of the infused catecholamines on the pulmonary capacity vessels seemed to dominate over volume changes secondary to systemic effects of the drugs. Our findings are thus in agreement with those Hauge *et al.* (1967) reported from isolated perfused lungs. In contrast to our findings Feelay *et al.* (1963) observed in intact dogs that the systemic effects of catecholamines predominated with increased pulmonary blood volume as a consequence.

As in the present experiments pulmonary blood volume did not increase during catecholamine infusions, although the vascular distending pressures were elevated, active vasoconstriction must have taken place. It thus appears that the pulmonary vascular bed can resist distension with unchanged, or even reduced resistance.

It has been debated whether recruitment of new vessels or distension of already open ones, are the important determinant for pulmonary blood volume as intravascular pressure change (Permutt *et al.* 1969). A large fraction of the pulmonary vascular resistance is located in the arteries. Therefore, with reduced vascular resistance, as seen during infusion of adrenalin (Fig. 1 a), a pressure elevation must have prevailed throughout the arterial tree. Since there was no increase in pulmonary blood volume in this situation significant vascular recruitment can apparently not have taken place. It follows that recruitment is unlikely to be an universal determinant for blood volume in cat lungs *in situ*, as it may be in dog lungs according to Permutt *et al.* (1969).

When compared to the values from the control group the animals given adrenalin for 28 min had a 4% increase in extravascular lung water content, whilst the animals given noradrenalin for the same period showed a small decrease in this parameter. The difference between the groups of infused animals in this respect was significant,  $p=0.026$ . Changes in extravascular water content in the lungs might reflect changes in hydrostatic capillary pressure. This had been demonstrated in isolated perfused rabbit lungs (Aarseth and Waaler

## The Importance of Asphyxia for the Development of Diving Bradycardia in Ducks

By

ARNOLDUS SCHYTTÉ BLIX

Received 19 February 1975

---

### Abstract

---

BLIX, A. S. *The importance of asphyxia for the development of diving bradycardia in ducks.*  
Acta physiol. scand. 1975. 95. 41-45

Upon submersion, the domestic duck normally displays prompt but modest cardiac depression. Such, after short delay is transformed into pronounced state of bradycardia. The present study has revealed that arterial asphyxia is prerequisite for the reinforcement of the initial slight bradycardia.

---

Once diving animal has overcome the immediate danger of drowning upon water immersion by cessation of breathing, it has to encounter the thereby incurred risk of asphyxiation. It is known that the most prominent adjustment displayed by diving vertebrates during such underwater episodes is their unusual ability to redistribute their blood in a way which greatly delays the exposure of brain and myocardium to asphyxia (Irving 1938). In principle this implies transformation of their cardiovascular system into "heart-brain-lung preparation" (Folkow 1968), delivering almost the total oxygen reserves in blood and lungs to these vitally important tissues. The integration of the various mechanisms initiating and reinforcing this neurogenic response (*i.e.* vasoconstriction and bradycardia) are, on the other hand, in several respect poorly understood. Hollenberg and Uvnäs (1963), Jones and Purves (1970), and Holm and Sørensen (1972) have investigated the consequences of carotid body denervation upon the cardiac responses to head immersion in the duck. They suggest on the basis of their results that the arterial chemoreceptors are mainly responsible for the cardiovascular responses to diving. It can be argued, however that the procedures used may not have included denervation of all chemosensitive receptors, and/or that the denervation has affected also other types of receptors (*e.g.* baroreceptors) that may be of great importance for the cardiovascular adjustments during diving. In order to get additional information about the importance of peripheral chemoreceptors for the establishment of the diving response, an experimental set up has been designed in which ducks can be exposed to head immersion and the associated apnoea without being exposed to asphyxia.



hour with this hypoxemia, indicating a strong precapillary vasoconstriction. The present experiments therefore lend no support to the hypothesis that direct vascular changes caused by the combined actions of hypoxemia and catecholamines are important etiological factors for the development of the wet lung syndrome. As has previously been shown, increased levels of catecholamines do play an indirect role in this connection, through interactions of the hormones with blood platelets (Bo and Hognestad 1971).

The conclusions from the present work are several. In situations where an increase in the level of circulating adrenaline is a predominant feature, a moderate increase in lung water content may result from the various circulatory adjustments to adrenaline, tending to give an increase in pulmonary capillary pressure. In situations where  $\alpha$ -receptors are markedly stimulated, and in situations with increased sympathetic activity combined with hypovolemia, precapillary vasoconstriction will however be overwhelming, thus preventing an increase in the hydrostatic lung capillary pressure. Other factors than direct adrenergic effects on the vessels must apparently be responsible for the accumulation of interstitial water in the lung tissue under such conditions. The key role played by blood platelets in the evolution of lung changes during hemorrhagic shock (Bo and Hognestad 1971) should be remembered here.

### References

- AARSETH, P. Reduction in pulmonary blood volume after a blood loss. *Acta physiol. scand.* 1970. **81** 459-469.
- AARSETH, P. and G. BO, Content of blood and of extravascular water in cat lungs during changes in blood volume. *Acta physiol. scand.* 1972. **85** 343-352.
- AARSETH, P. and B. A. WAALER, Interstitial water content in isolated rabbit lungs at different levels of capillary pressure. *Acta physiol. scand.* 1974. Suppl. 396. 76.
- AARSETH, P., G. BO and A. H. UGE, In preparation.
- BEAN, J. W. and D. L. BECKHAM, Centrogenic pulmonary pathology in mechanical head injury. *J. appl. Physiol.* 1969. **27** 807-812.
- BO, G. and J. HOGNESTAD, The role of the adrenal medulla in posthemorrhagic pulmonary hypertension. *Acta physiol. scand.* 1971. **83** 124-132.
- OLANDER, O. The range of control exercised by the "sympathico-adrenal system". *Acta physiol. scand.* 1954. Suppl. 116.
- ERSOZ, N. and S. C. FINESTONE, Adrenaline induced pulmonary oedema and its treatment. *Brit. J. Anaesth.* 1971. **43** 709-712.
- FELLY, J. W., T. D. LEE and W. R. MILNOR, Active and passive components of pulmonary vascular response to vasoactive drugs in the dog. *Amer. J. Physiol.* 1963. **205** 1193-1199.
- H. UGE, A. and N. C. STUBB, Prevention of hypoxic vasoconstriction in cat lung by histamine-releasing agent 48/80. *J. appl. Physiol.* 1969. **26** 693-699.
- HAUGE, A., P. M. K. LUNDE and B. A. WAALER, Effects of catecholamines on pulmonary blood volume. *Acta physiol. scand.* 1967. **70** 323-333.
- KARLSEN, J., G. BO and P. AARSETH, The volumes of blood and of extravascular water in the lungs of cats during periods of acute hypoxemia. *Microvasc. Res.* 1973. **6** 256.
- LUBADA, A. A., Mechanism of neurogenic pulmonary edema. *Amer. J. Cardiol.* 1967. **20** 66-68.
- MOORE, F. D., J. H. LYONS, E. C. PIERCE, A. P. MORGAN, P. A. DEINER, J. D. MACARTHUR and G. J. DAMEN, *Post-traumatic pulmonary insufficiency*. W. B. Saunders Company Philadelphia 1969.
- PERMUTT, S., P. CALDWELL, A. M. TIERI, W. H. P. LAM, T. SASAMORI and K. J. LI, Recruitment and distensibility in the pulmonary vasculature. I. *The pulmonary circulation and interstitial space*. Ed. by A. P. Fishman and H. H. Hecht. The University of Chicago Press, Chicago 1969.
- WHAYNE, Th. F. and J. W. SEVERINGHOUSE, Experimental hypoxia pulmonary edema in the cat. *J. appl. Physiol.* 1968. **25** 729-734.

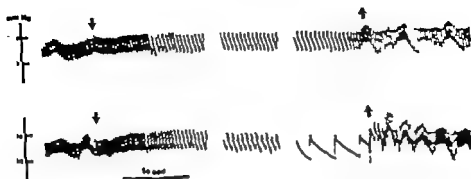


Fig. 1. The typical blood pressure response to 1 min head immersion in ducks. The recordings were made at the beginning, after 30 s, and at the end of the dive. *Upper tracing*: The response to non-asphyxic submersion (like the lower tracing of Fig. 2). *Lower tracing*: The response to submersion with asphyxia, the lungs being perfused by gas mixture of 3%  $O_2$  in  $N_2$ .

Table I). Largely normal diving responses were also obtained when the lungs were perfused with  $O_2$ - $N_2$  mixture of low oxygen concentration (3%) provided the animal did not struggle (Fig. 3). The delay of the response in this experiment might be due to the fact that some oxygen was supplied. Perfusion with pure nitrogen led to such vigorous struggling that the diving response was partly masked.

If the lungs were instead "ventilated" by a retrograde influx of 20% oxygen in nitrogen to the air sac during submersion, resulting in a maintenance of normal or slightly super normal arterial  $PO_2$  (usually associated with slight hypocapnia, as revealed by an arterial pH of  $7.49 \pm 0.03$  (S.E.,  $n = 9$ ), they all responded to head immersion with the usual apnoea and a quiet behaviour. The cardiovascular response to submersion, was, however, now strikingly different from that of a normal dive. As seen from Fig. 2, the heart rate fell almost instantaneously upon submersion to about 60% of the pre-dive value, but did not further decrease as long as the oxygen delivery via the air sac continued, no matter how long the dive continued. Conversely upon emersion the heart rate promptly returned to the pre-dive level without the usual overshoot in terms of a transient pressure rise and tachycardia (Table I).

TABLE I Heart rate of ducks, before, after 60 s, and after 5 min of recovery from normal and non-asphyxic dives, all lasting for 90 s. The numbers represent means and standard error of the means (S.E.). Included are also the calculated per cent of pre-dive value (%), for each group.

	Before dive M $\pm$ S.E.	After 60 s dive		After 5 min recovery	
		M $\pm$ S.E.	% $\pm$ S.E.	M $\pm$ S.E.	% $\pm$ S.E.
Normal	251 $\pm$ 40 (5)	24 $\pm$ 4 (5)	9 $\pm$ 2 (5)	327 $\pm$ 29 (5)	140 $\pm$ 23 (5)
Non-asphyx	233 $\pm$ 30 (9)	132 $\pm$ 14 (9)	59 $\pm$ 3 (9)	247 $\pm$ 16 (9)	113 $\pm$ 5 (9)



Fig. 1 Arterial blood pressure of a duck breathing air at surface position. Unidirectional, retrograde and nonpulsatile left lung ventilation with air (inlet pressure 1.5 cm H<sub>2</sub>O), performed between arrows.

### Material and methods

Eight domestic (Pekin) ducks of both sexes, weighing 2–3 kg, none of which displayed spontaneous breathing through their tracheostomy while submerged, were used in this study. The trachea was cannulated with a 4.5 mm Floetex® endotracheal tube, and an intravascular catheter (PP 100) was inserted into the brachial artery. Another polyethylene catheter (inner diameter = 3 mm) with side holes was advanced through the thoracic wall into the right posterior thoracic air sac, care being taken to achieve an air-tight seal around the cannula and to avoid bleeding (post mortem inspection). All cannulations were performed under local (Xylorcin® 1%) anaesthesia.

During the experiment the animal was restrained to a board ventral side down. The arterial catheter was used for pressure recording (Statham transducer P23H) and blood sampling and arterial PO<sub>2</sub> and pH were analysed by use of specific Radiometer electrodes. The air sac catheter was connected to a modified spirometer whereby the animal could be oxygenated by sending a stream of oxygen (~90%) in nitrogen in a retrograde direction through the lung, at a nonpulsatile inlet pressure of 1.5 cm H<sub>2</sub>O. During as simulated by gently tilting the board so that the head was submerged in a water pool (the water temperature is kept at 25°C). In order to secure unidirectional gas flow through the system during the dive, a one-way valve was connected to the end of the tracheal tube at the very moment of submersion. After recording the normal cardiovascular responses to head immersion (*i.e.* without oxygenation ("diving with asphyxia"), the procedures were repeated while the lungs were kept oxygenated through the air sac cannula ("dive without asphyxia").

### Results

During normal breathing in the pre-dive situation, onset of the unidirectional, retrograde and non-pulsatile pulmonary "ventilation" did not *per se* affect significantly either blood pressure, heart rate or respiratory rate (Fig. 1). Diving, without this retrograde pulmonary ventilation resulted in typical respiratory and cardiovascular responses to submersion with an initial modest bradycardia that was greatly reinforced during prolonged immersion (Fig. 2).

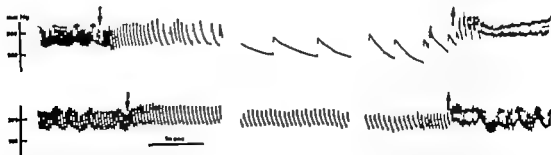


Fig. 2. The typical blood pressure response to 2 m head immersion in a duck. The recordings were made at the beginning, after 60 s, and at the end of the dive. *Upper tracing* The normal response (without any kind of ventilation). *Lower tracing* The response of the same animal, while being ventilated as described for Fig. 1 (with oxygen/nitrogen mixture—a non-asphyxic dive).

- KOLLEHNSG, N. K. and B. UYVÄL, The role of the cardiovascular response in the resistance to asphyxia of *vis adiver*. *Acta physiol. scand.* 1963. 38. 150-161.
- TOLM, B. and B. C. SANDERSON, The role of the carotid body in the diving reflex in the duck. *Resp. Physiol.* 1972. 15. 303-309.
- LYNCH, L., Changes in the blood flow through the brain and nescies during the arrest of breathing. *Amer. J. Physiol.* 1938. 122. 207-214.
- OWEN, D. R. and M. J. PURVES, The carotid body in the duck and the consequences of its denervation upon the cardio response to immersion. *J. Physiol. (Lond.)* 1970. 211. 279-294.
- LEWIS, L.-M. and M. ROSEY, Vagal afferent activities related to the respiratory cycle in the duck. sensitivity to mechanical chemical and electrical stimuli. *Resp. Physiol.* 1974. 22. 41-56.
- SHIMO, B. and P. TANOCHI, Studies on left ventricular receptors, signalling in non-sensitized afferents. *Acta physiol. scand.* 1972. 85. 145-163.

### Discussion

The essential finding of the present investigation is that ducks display a prompt, but quite modest bradycardia response when exposed to submersion provided arterial hypoxia is avoided. This suggests that the initial bradycardia is independent of asphyxia. Bradycardia also occurs, although to a lesser extent, even when ducks are artificially ventilated with air at normal tidal volumes during diving (Feigl and Folkow 1963). However it has been shown in geese (Cohn *et al.* 1968) and we have found it to be true of some ducks also that both the initial and the delayed bradycardia component are closely associated with apnoea. It seems likely therefore, that the first event in diving is an afferent input from peripheral receptors (Andersen 1963) which induces an apnoeic state. This, in turn, causes a prompt but limited bradycardia, which is slightly enhanced due to the absence of an afferent input from lung receptors, like those described *e.g.* by Leitner and Roumy (1974).

It has been shown previously (Blix and Berg 1974), that the delayed intensification of the initial diving bradycardia can be elicited by pure arterial hypoxia (even combined with a slight hypocapnia). This could indicate that the pronounced bradycardia arises as a primary chemoreceptor response (Daly and Scott 1963). However Blix *et al.* (1974) and Andersen and Blix (1974) have demonstrated that the delayed intensification of the bradycardia does not occur if the peripheral neurogenic vasoconstriction is abolished. These findings may point to an important involvement also of high- and low pressure mechanoreceptors in the production of the intensified diving bradycardia in ducks. In respect of this, the left ventricular "volume receptors" described by Öberg and Thorén (1972) may be of great relevance.

In conclusion, the delayed pronounced bradycardia, unlike the initial cardiac retardation of diving ducks, seems to be closely associated with arterial asphyxia. The manifestation of the response is, however in some manner dependent upon the chemoreceptor initiated peripheral vasoconstriction.

Sponsored by the Norwegian Research Council for Science and the Humanities.

I wish to thank Mr K. A. Stokkan and Miss B. Larsen for their ample assistance during the experiments.

### References

- ANDERSEN, H. T. The reflex nature of the physiological adjustments to diving, and their afferent pathways. *Acta physiol. scand.* 1963 38 263-273.
- ANDERSEN, H. T. and A. S. BLIX. Pharmacological exposure of components in the monoaminic control of the diving reflex. *Acta physiol. scand.* 1974 90 381-386.
- BLIX, A. S. and T. BERG. Arterial hypoxia and the diving responses of ducks. *Acta physiol. scand.* 1974 92, 566-568.
- BLIX, A. S., E. L. GAUTVIK and H. RØRUM. Aspects of the relative roles of peripheral vasoconstriction and vagal bradycardia in the establishment of the diving reflex in ducks. *Acta physiol. scand.* 1974 90 289-296.
- COHN, J. E., J. KROG and R. SHANMUG. Cardiopulmonary responses to head immersion in domestic geese. *J. appl. Physiol.* 1968, 25 36-41.
- DALY, M. de BURGH and M. J. SCOTT. The cardiovascular responses to stimulation of the carotid body chemoreceptors in the dog. *J. Physiol. (Lond.)* 1963 165 179-197.
- FRIGL, E. and B. FOLKOW. Cardiovascular responses to diving and during brain stimulation in ducks. *Acta physiol. scand.* 1963, 37 99-110.
- FOLKOW, B., Circulatory adaptations to diving in ducks. *Proc. Intern. Union. Physiol. Sci.* 1968 6 23-4.

to literature that NA, and to some extent A, are also removed from the pulmonary circulation (Eisenmann *et al.* 1964 Hughes *et al.* 1969 Gilin and Vane 1968 Mathé *et al.* 1975), in various experimental animals as well as in man (Blron *et al.* 1969 Gillis *et al.* 1972).

The purpose of the present investigation was to study the extent and degree of specificity of the acute removal of circulating tritium-labelled NA from the blood stream, during one circulation through the lungs, and through a well-defined systemic vascular bed (forearm vasculature), in man. A mixture of  $^3\text{H}$ -(-)-NA,  $^{14}\text{C}$ -inulin and  $^{125}\text{I}$ -albumin was injected proximally to the vascular bed under study and serial blood samples were rapidly drawn from the effluent distally to it. The disappearance of  $^3\text{H}$  relatively to  $^{125}\text{I}$  was taken as a measure of total (unspecific + specific) removal of  $^3\text{H}$  NA, that of  $^{14}\text{C}$  relatively to  $^{125}\text{I}$  as a measure of filtration + diffusion and that of  $^3\text{H}$  relatively to  $^{14}\text{C}$  as a measure of specific removal of  $^3\text{H}$ -NA.

## Material and Methods

### Infusion of markers sampling of blood

18 healthy male subjects, 20-35 years old, whose informed consent was obtained, were used for the experiment. T block thyroid uptake of the  $^{125}\text{I}$  used in the experiments, 50  $\mu\text{g}$  NaI was given orally the day before.

In the study of removal of circulating NA during single passage through the pulmonary vascular bed (118 expts) the bolus injection of the mixture of  $^3\text{H}$  NA,  $^{14}\text{C}$ -inulin and  $^{125}\text{I}$ -albumin (see below) was made through catheter introduced percutaneously through the right antecubital vein and under fluoroscopic control guided into the right atrium near the tricuspid valve. For sampling blood after one circulation through the lungs a second percutaneous catheter was introduced into the right brachial artery.

In the study of systemic vascular bed (8 expts) the injection was made through catheter in the brachial artery and blood was sampled from catheter introduced in the distal direction into a deep vein of the forearm. A cuff was applied around the wrist and inflated to pressure of 220 mm Hg to exclude hand circulation. With this arrangement blood sampled from the deep vein mainly represents effluent from forearm tissue (Coles *et al.* 1958).

Immediately before injection, the three radioactive compounds, 10  $\mu\text{Ci}$   $^3\text{H}$ -(-)-NA (New England Nuclear sp. 675 Ci/mmole), 5  $\mu\text{Ci}$   $^{14}\text{C}$ -labelled inulin (New England Nuclear isobutyl-cuorbyl- $^{14}\text{C}$ , sp. 1.3 mCi/ $\mu\text{Ci}$ ) and 20-30  $\mu\text{Ci}$   $^{125}\text{I}$ -labelled human serum albumin (human serum albumin, Kabi, labelled with  $^{125}\text{I}$ , New England Nuclear the extent of labelling as approximately one atom of iodine per protein molecule, and the  $^{125}\text{I}$ / $^{127}\text{I}$  ratio was about  $10^{-4}$  the labelled albumin was separated from contaminating unbound moieties by gel filtration on Sephadex), were mixed and diluted to 5 ml with saline. After removing

5 ml of control blood 4.5 ml volume of the mixture was rapidly injected into the right atrium, or into the brachial artery respectively. A series of six 5 ml samples of blood were then drawn, at the respective sampling point, with about 15 intervals between successive samples, starting 5 or 15 s after the injection, in the experiments with the systemic or the pulmonary vascular bed, respectively.

### Analytical

All blood samples were immediately chilled with ice, after mixing with 1 ml 0.5 M NaCl containing 0.1 EDTA to prevent clotting and 10  $\mu\text{g}$  unlabelled NA to protect the labelled NA in the samples.

To increase the accuracy of the estimation of the degree of metabolic degradation of the injected  $^3\text{H}$  NA during the single passage through the two vascular beds tested, 0.5 ml volume of the injection mixture remaining in the syringe was diluted ten-fold with saline containing 0.1 EDTA and 10  $\mu\text{g}$  unlabelled NA per ml, and 0.5 ml of this solution was added to each of the two control blood samples drawn immediately before the injection. These controls were carried through all the various preparative and analytical steps together with the five 'test' samples.

After centrifugation for ten min in chilled laboratory centrifuge (Christ, swing-out buckets, 3 000  $\text{r/min}$ ) to remove blood cells, 2-3 ml volume of plasma was pipetted off. One ml of this was used for determination of total  $^{125}\text{I}$  activity. To one ml of the remainder was added 0.1 ml 4 M perchloric acid to precipitate proteins, including  $^{125}\text{I}$ -labelled albumin. After centrifugation the protein-free supernatant was used for determination

From the Department of Physiology Karolinska Institutet, the Department of Clinical Physiology Karolinska Sjukhuset and the Department of Internal Medicine Huddinge Sjukhus, Stockholm, Sweden

## Specific and Unspecific Removal of Circulating Noradrenaline in Pulmonary and Systemic Vascular Beds in Man

By

LENNART STJÄRNE, LENNART KAISER, ALEXANDER MATIĆ and GUNNAR BIRKE

Received 21 February 1975

---

### Abstract

---

STJÄRNE, L., L. KAISER, A. MATIĆ and G. BIRKE. *Specific and unspecific removal of circulating noradrenaline in pulmonary and systemic vascular beds in man* Acta physiol. scand. 1975 95 46-53

In order to study the extent and degree of specificity of the removal of circulating noradrenaline (NA) from the blood stream, during a single passage through the vascular beds of the lungs and of the forearm musculature of unanaesthetized healthy young men the following radiometric method was used. A mixture of H NA with  $^{14}\text{C}$  inulin and  $^{125}\text{I}$  labelled human serum albumin was injected proximally to the bed under study: six serial blood samples were drawn distally to it with about 15 s interval, and the disappearance of H NA relatively to that of  $^{14}\text{C}$  inulin and  $^{125}\text{I}$  albumin was determined. H NA was found to be removed from the blood stream in both these vascular beds. However in the forearm the removal of H NA was much greater and apparently to a large extent due to specific trapping in the tissues, since the clearance of H NA over the vascular bed of the forearm markedly exceeded that of  $^{14}\text{C}$  inulin. During passage through the lungs removal of H NA was less marked and apparently largely unspecific, since it was initially essentially equal to that of  $^{14}\text{C}$  inulin. This suggests that removal of circulating ergones of small molecular size, from the blood stream passing through the lungs, may occur initially by unspecific filtration/adsorption. Their subsequent fate would depend on whether the extraluminal tissues, supplied by the lung circulation, possess specific mechanisms for concentrating and/or sequestering each particular compound.

---

As early as 1905 Elliott concluded that "adrenalin disappears in the tissues which it excites. Later experiments have confirmed that circulating adrenalin (A) (Burn 1932) and noradrenaline (NA) (Celander and Melander 1955) are extensively removed from the circulation during single passages through various vascular beds: both A and NA have been shown to have a half-life in the circulation of less than 20 s, or less than one circulation time (Ferreira and Vane 1967). While several systemic vascular beds have been found to be much more efficient than the pulmonary vascular bed in removing circulating catecholamines (Elliott 1905 Celander and Melander 1955 Ferreira and Vane 1967), there are reports in

literature that NA, and to some extent A, are also removed from the pulmonary circulation (Elsom *et al.* 1964, Hughes *et al.* 1969, Ginn and Vane 1968, Mathé *et al.* 1975), various experimental animals as well as in man (Biron *et al.* 1969, Gillis *et al.* 1972).

The purpose of the present investigation was to study the extent and degree of specificity for acute removal of circulating tritium-labelled NA from the blood stream, during one circulation through the lungs, and through a well-defined systemic vascular bed (forearm circulation), in man. A mixture of  $^3\text{H}$ -(-)-NA,  $^{14}\text{C}$ -inulin and  $^{125}\text{I}$ -albumin was injected proximally to the vascular bed under study and serial blood samples were rapidly drawn in the effluent distally to it. The disappearance of  $^3\text{H}$  relatively to  $^{125}\text{I}$  was taken as a measure of total (unspecific + specific) removal of  $^3\text{H}$  NA, that of  $^{14}\text{C}$  relatively to  $^{125}\text{I}$  as a measure of filtration + diffusion and that of  $^3\text{H}$  relatively to  $^{14}\text{C}$  as a measure of specific removal of  $^3\text{H}$  NA.

### Material and Methods

#### Source of markers, sampling of blood

Healthy male subjects, 20–35 years old, whose informed consent was obtained, were used for the experiments. T block thyroid uptake of the  $^{125}\text{I}$  used in the experiments, 30 mg NaI was given orally the day before.

In the study of removal of circulating NA during a single passage through the pulmonary vascular bed (8 expts) the bolus injection of the mixture of  $^3\text{H}$  NA,  $^{14}\text{C}$ -inulin and  $^{125}\text{I}$ -albumin (*see below*) as made through catheter introduced percutaneously through the right antecubital vein and under fluoroscopic control guided into the right atrium near the tricuspid valve. For sampling blood after one circulation through the lungs, second percutaneous catheter was introduced into the right brachial artery.

In the study of systemic vascular bed (8 expts) the injection was made through catheter in the brachial artery and blood was sampled from catheter introduced in the distal direction into deep vein of the same arm. A cuff was applied around the wrist and inflated to pressure of 220 mm Hg to exclude hand circulation. With this arrangement blood sampled from the deep vein mainly represents effluent from muscle tissue (Coles *et al.* 1953).

Immediately before injection, the three radioactive compounds, 10  $\mu\text{Ci}$   $^3\text{H}$ -(-)-NA (New England Nuclear sp. 6.75 Ci/mmol), 5  $\mu\text{Ci}$   $^{14}\text{C}$ -labelled inulin (New England Nuclear inulin-carboxyl- $^{14}\text{C}$ , sp. 1.3 mCi/g) and 20–30  $\mu\text{Ci}$   $^{125}\text{I}$ -labelled human serum albumin (Norman serum albumin, Kabi, labelled with  $^{125}\text{I}$ , New England Nuclear) the extent of labelling was approximately one atom of iodine per protein molecule, and the  $^{125}\text{I}$ / $^{14}\text{C}$  ratio was about  $10^{-4}$ ; the labelled albumin was separated from contaminating, unbound isotopes by gel filtration on Sephadex, were mixed and diluted to 5 ml with saline. After removing 2–5 ml of control blood, 4–5 ml volume of the mixture was rapidly injected into the right atrium, or into the brachial artery respectively. A series of six 5 ml samples of blood were then drawn, at the respective sampling point, with about 15 intervals between successive samples, starting 5 or 15 s after the injection, in the experiments with the systemic or the pulmonary vascular bed, respectively.

#### Assay method

All blood samples were immediately chilled with ice, after mixing with 1 ml 0.9% NaCl containing 0.1 EDTA to prevent clotting and 10  $\mu\text{g}$  unlabelled NA to protect the labelled NA in the samples.

To increase the accuracy of the estimation of the degree of metabolic degradation of the injected  $^3\text{H}$  NA during the single passage through the two vascular beds tested, 0.5 ml volume of the injection mixture remaining in the syringe was diluted ten-fold with saline containing 0.1% EDTA and 10  $\mu\text{g}$  unlabelled NA per ml, and 0.5 ml of this solution was added to each of the two control blood samples drawn immediately before the injection. These controls were carried through all the various preparative and analytical steps together with the five 'test' samples.

After centrifugation for 10 min in chilled laboratory centrifuge (Christ, swing-out buckets, 3000  $\times g$ , max.) to remove blood cells, 2–3 ml volume of plasma was pipetted off. One ml of this was used for determination of total  $^3\text{H}$  activity. To one ml of the remainder was added 0.1 ml 4 M perchloric acid to precipitate protein, including  $^{125}\text{I}$ -labelled albumin. After centrifugation the protein-free supernatant was used for determination



## Specific and Unspecific Removal of Circulating Noradrenaline in Pulmonary and Systemic Vascular Beds in Man

By

LENNART STJÄRNE, LENNART KAUSER, ALEXANDER MATHÉ\* and GUNNAR BIRKE

Received 21 February 1975

### Abstract

STJÄRNE, L., L. KAUSER, A. MATHÉ and G. BIRKE. *Specific and unspecific removal of circulating noradrenaline in pulmonary and systemic vascular beds in man* Acta physiol scand. 1975 95 46-53

In order to study the extent and degree of specificity of the removal of circulating noradrenaline (NA) from the blood stream, during a single passage through the vascular beds of the lungs and of the forearm musculature of unanaesthetized, healthy young men, the following radiometric method was used. A mixture of H NA with  $^4\text{C}$  inulin and  $^{125}\text{I}$  labelled human serum albumin was injected proximally to the bed under study; six serial blood samples were drawn distally to it, with about 15 s interval, and the disappearance of H NA relatively to that of  $^4\text{C}$  inulin and  $^{125}\text{I}$ -albumin was determined. H NA was found to be removed from the blood stream in both these vascular beds. However in the forearm the removal of H NA is much greater and apparently to a large extent due to specific trapping in the tissues, since the clearance of H NA over the vascular bed of the forearm markedly exceeded that of  $^4\text{C}$  inulin. During passage through the lungs removal of H NA was less marked, and apparently largely unspecific, since it was (almost) essentially equal to that of  $^4\text{C}$  inulin. This suggests that removal of circulating ergones of small molecular size, from the blood stream passing through the lungs, may occur initially by unspecific filtration/diffusion. Their subsequent fate would depend on whether the extraluminal tissues, supplied by the lung circulation, possess specific mechanisms for concentration and/or inactivating each particular compound.

As early as 1905 Elliott concluded that "adrenalin disappears in the tissues which it excites. Later experiments have confirmed that circulating adrenaline (A) (Burn 1932) and noradrenaline (NA) (Celander and Melander 1955) are extensively removed from the circulation during single passages through various vascular beds: both A and NA have been shown to have a half life in the circulation of less than 20 s, or less than one circulation time (Ferreira and Vane 1967). While several systemic vascular beds have been found to be much more efficient than the pulmonary vascular bed in removing circulating catecholamines (Elliott 1905, Celander and Melander 1955, Ferreira and Vane 1967) there are reports in

\*Present address: Laboratory of Biogenic Amines and Allergy, Boston University Medical Center, Boston, Mass. 02118, USA

## REMOVAL OF CIRCULATING NA IN MAN

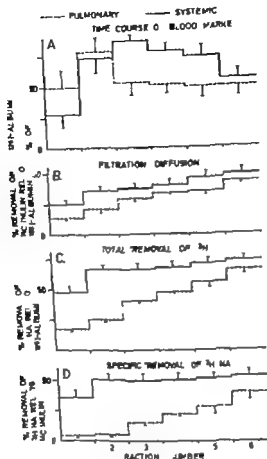


Fig. 1. A. Time course of appearance of  $^{251}\text{I}$ -labelled albumin, used as a marker for (non-diffusible compounds as) "blood" at the respective sampling points (for pulmonary circulation: brachial artery; for systemic circulation: deep vein of the same arm, into the brachial artery of which the injection was made). Means  $\pm$  S.E. (in panels A-D). B. Per cent removal of  $^{14}\text{C}$ -urea, in relation to  $^{251}\text{I}$ -albumin. If the  $^{14}\text{C}/^{251}\text{I}$  ratio in the test sample equals that of the control, that would imply 0% removal of  $^{14}\text{C}$ -urea. If  $^{14}\text{C}$  in the test sample falls to 0, removal of  $^{14}\text{C}$ -urea would be 100%. Removal of  $^{14}\text{C}$ -urea relatively to  $^{251}\text{I}$ -albumin is regarded as a measure of elimination of solutes of small molecular size, by filtration + diffusion. C. Per cent removal of  $^3\text{H}$ -NA, in relation to  $^{251}\text{I}$ -albumin (same type of calculation as above), regarded as a measure, in the first 2-3 fractions, of total removal of  $^3\text{H}$ -NA from the blood stream, during one passage through the respective vascular bed. D. Per cent removal of  $^3\text{H}$ -NA, in relation to  $^{14}\text{C}$ -urea (same type of calculation as in B). If the  $^3\text{H}$ -NA/ $^{14}\text{C}$ -urea ratio in the test sample equals that of the control (in which case the change in  $^3\text{H}$ -NA/ $^{251}\text{I}$ -albumin ratio, after passage through the respective vascular bed, would equal that as  $^{14}\text{C}$ -urea/ $^{251}\text{I}$ -albumin ratio), total removal of  $^3\text{H}$ -NA would equal total removal of  $^{14}\text{C}$ -urea. Since  $^{14}\text{C}$ -urea-removal is assumed to occur by unspecific mechanisms,  $^3\text{H}$ -NA removal would in that case also be entirely unspecific (specific removal of  $^3\text{H}$ -NA would be 0%). If the  $^3\text{H}$ -NA/ $^{14}\text{C}$ -urea ratio is reduced to, e.g. one half, this would imply that  $^3\text{H}$ -NA is removed twice as efficiently as  $^{14}\text{C}$ -urea, of the total amount of  $^3\text{H}$ -NA eliminated from the blood stream 50% would be removed 'unspecifically' (by filtration/diffusion), and 50% 'specifically' by additional specific trapping in the tissues.

$$\left[ -1 \frac{\left[ \frac{^{14}\text{C}}{^{251}\text{I}} \right]_{\text{Test}}}{\left[ \frac{^{14}\text{C}}{^{251}\text{I}} \right]_{\text{Control}}} \right] 100$$

of total H and  $^{14}\text{C}$ , and for cation exchange column chromatography (Amberlite CG 120, Type 2, 200–400 mesh, columns 6 cm in height and 0.5 cm in diameter eluant 1 M hydrochloric acid), for separation of labelled NA from its various metabolites (cf Stjärne 1966).

To determine H and  $^{14}\text{C}$  0.4 ml aliquots of the different fractions were counted (in 70 ml of a 7:3 mixture of toluene with ethanol, containing 4 g of PPO and 0.1 g of POPOP per liter toluene) H and  $^{14}\text{C}$  in the mixture were determined by simultaneous dual channel counting for 10 min, with correction for 'spillover'. In an ABAC SL40 Intertechnique Liquid Scintillation Spectrometer  $^{125}\text{I}$  activity was determined with a scintillation detector the pulse height analyzer of which was adjusted to a maximum sensitivity at 0.69 MeV.

#### Premises

The aim of the experiments was to study the acute removal of H NA, during one passage through different vascular beds. H NA was injected together with  $^{125}\text{I}$  labelled albumin, used as a marker for substances not leaving the blood stream, and  $^{14}\text{C}$  labelled inulin, as a marker for substances freely diffusible and filterable, through the capillary walls.

The fall in H/ $^{125}\text{I}$  ratio, in blood sampled distally to the vascular bed under study when compared to that in control blood samples in which the isotope mixture was added *in vitro*, should thus, independently of the absolute levels of H or  $^{125}\text{I}$  reflect total removal of H NA from the blood stream. The fall in  $^{14}\text{C}/^{125}\text{I}$  ratio should reflect unspecific removal of diffusible solutes from the blood stream. And, provided that H NA and  $^{14}\text{C}$  inulin disappear from the blood stream to an equal extent in tissues lacking specific trapping mechanisms for H NA, a reduction in the original  $^{125}\text{I}/^{14}\text{C}$  ratio should give a measure of the specific removal of H NA.

## Results

#### Time course of appearance of $^{125}\text{I}$ at sampling points

The results in this respect are expressed in relative terms. The peak level of  $^{125}\text{I}$  in each experiment was given the value of unity and the  $^{125}\text{I}$  activity in the other sampling periods of that experiment were normalized with respect to this. As shown in Fig. 1A, the peak was most frequently reached in the second sampling period in the pulmonary circulation and in the third period in the systemic bed under study.

Since these peak periods reflect mean transit time it follows that mainly data obtained from them are of relevance to the problem under study since they represent removal during a single passage through the respective vascular bed.

#### Changes in the H/ $^{125}\text{I}$ ratio

During a single passage through the forearm vascular bed the H/ $^{125}\text{I}$  ratio was immediately strongly reduced. In the first few samples taken after one circulation through the pulmonary bed it fell only moderately (the difference from the corresponding values in the systemic bed during each of the first four sampling periods was highly significant,  $p < 0.001$   $n = 13$  B), when compared to *in vitro* controls, but in later samples it progressively declined to approach the level observed in the samples from the systemic bed. Thus the total removal of H NA was 65–70% (from the second period and onwards) during one passage through the bed of the forearm, while total removal of H NA in the pulmonary circulation was initially much lower progressively increasing from 17.5% in the first sample to 65% in the last (Fig. 1C where the results are plotted as the deficit in H relatively to  $^{125}\text{I}$  or per cent total removal of H NA).

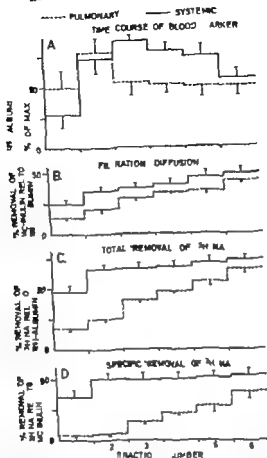


Fig. 1. A. Time course of appearance of  $^{125}\text{I}$ -labelled albumin, used as marker for (non-diffusible compounds as) "blood" at the respective sampling points (for pulmonary circulation, brachial artery for systemic circulation, deep vein of the arm vein, into the brachial artery of which the injection was made). Means  $\pm$  S.E. (in panels A-D). B. Per cent removal of  $^{14}\text{C}$ -astrotin in relation to  $^{125}\text{I}$ -albumin. If the  $^{14}\text{C}$ -astrotin in the test sample equals that of the control, that would imply 0% removal of  $^{14}\text{C}$  locally. If  $^{14}\text{C}$  in the test sample falls to 0, removal of  $^{14}\text{C}$ -astrotin would be 100%. Removal of  $^{14}\text{C}$ -astrotin relatively to  $^{125}\text{I}$ -albumin is regarded as a measure of elimination of solutes of small molecular size, by filtration + diffusion. C. Per cent removal of  $^3\text{H}$ -NA, in relation to  $^{125}\text{I}$ -albumin (same type of calculation as above), regarded as a measure, at the first 2-3 fractions, of total removal of  $^3\text{H}$ -NA from the blood stream, during its passage through the respective vascular bed. D. Per cent removal of  $^3\text{H}$ -NA, in relation to  $^{14}\text{C}$ -astrotin (same type of calculation as in B). If the  $^3\text{H}$ -NA/ $^{14}\text{C}$ -astrotin ratio in the test sample equals that of the control (i.e. back into the change in  $^3\text{H}$ -NA/ $^{125}\text{I}$ -albumin ratio, after passage through the respective vascular bed, would equal that in  $^{14}\text{C}$ -astrotin/ $^{125}\text{I}$ -albumin ratio), total removal of  $^3\text{H}$ -NA would equal total removal of  $^{14}\text{C}$ -astrotin. Since  $^{14}\text{C}$ -astrotin-removal is assumed to occur by unspecific mechanisms,  $^3\text{H}$ -NA removal could in that case also be entirely unspecific (specific removal of  $^3\text{H}$ -NA would be 0%). If the  $^3\text{H}$ -NA/ $^{14}\text{C}$ -astrotin ratio is reduced to 1/2 one half, that would imply that  $^3\text{H}$ -NA is removed twice as efficiently as  $^{14}\text{C}$ -astrotin, of the total amount of  $^3\text{H}$ -NA eliminated from the blood stream 50% would be removed 'unspecifically' (by filtration/diffusion), and 50% 'specifically' (by additional specific trapping in the tissues).

$$\left[ \frac{\left[ \frac{^{14}\text{C}}{^{125}\text{I}} \right]_{\text{Test}}}{\left[ \frac{^{14}\text{C}}{^{125}\text{I}} \right]_{\text{Control}}} \right] \cdot 100$$

		ON		EFFLUENT			ON		EFFLUENT
		dpm <sub>TOT</sub>		dpm <sub>TOT</sub>			dpm <sub>TOT</sub>		dpm <sub>T</sub>
CONTROL	<sup>3</sup> H	55140	1250	2.2%	PERIOD 2	<sup>3</sup> H	86790	6113	7.0
	<sup>14</sup> C	70340	8430	81.5%		<sup>14</sup> C	33173	28422	85.6%

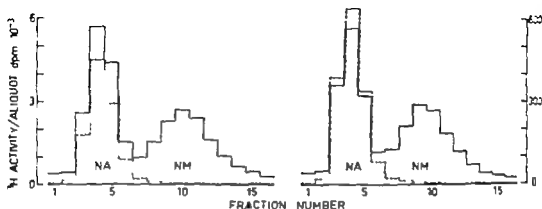


Fig. 2. Typical examples of ion exchange chromatograms of perchloric acid extracts of plasma from control and test samples. For further details see the Text.

#### Changes in $C/I$ ratio

A progressive fall in the  $^{14}C/I$  ratio reflecting the degree of non-specific 'removal' diffusible solutes from the blood stream, was observed in both the pulmonary and the systemic bed (Fig. 1 B). Although the removal of  $^{14}C$  inulin during a single passage through the lungs was smaller than that during one circulation through the forearm ( $p < 0.001$  pool data,  $n = 103$ ) the results still indicate that diffusible solutes to a considerable extent do leave the circulating blood stream in the vascular bed of the lungs as well as in that of the forearm.

#### Changes in $H/I$ ratio

The changes in  $H/I$  ratio in the two vascular beds were very markedly different ( $p < 0.05$  for the first three sampling periods,  $n = 13-18$  in each). After one passage through the forearm the fall in this ratio was about 50%, whereas passage through the lungs did initially, in the first two samples, not change the  $H/I$  ratio at all, when compared to *in vitro* controls. In later samples it progressively fell to approach the levels seen in the systemic bed (Fig. 1 D).

#### Identity of $^3H$ in blood samples

Ion exchange chromatographic analysis of perchloric acid extracts of plasma from blood taken from the sampling points gave exactly the same results as that of control blood samples to which the radioactive mixture was added *in vitro* (cf. under Methods). In all cases a single peak of  $^3H$  activity was obtained, exactly coinciding with the fluorescence peak of added carrier NA (Fig. 2). The effluent from the columns contained  $0.98 \pm 0.57\%$  ( $n = 4$ ) at  $1.75 \pm 0.91\%$  ( $n = 8$ ) of the added  $^3H$  activity in controls and test samples, respectively. A  $^3H$  activity peak was observed coinciding with normetanephrine. The total recovery of  $^3H$

in the  $^3\text{H}$  NA peak in the test samples was identical with that in the controls. It may be concluded that all of the  $^3\text{H}$  appearing at the respective sampling points, during a time of observation, represented unchanged  $^3\text{H}$  NA.

### Discussion

aim of the present study was to compare the acute removal of  $^3\text{H}$  NA from the blood, during one circulation, through the pulmonary and one systemic vascular bed, in

results clearly show that, in man,  $^3\text{H}$ -NA is removed from the blood stream in both vascular beds under study although much more promptly and efficiently from that of the forearm than from that of the lungs. That removal of catecholamines is more marked in the pulmonary bed has previously been observed in various experimental animals (Elliott 1905, Celander and Melander 1955, Ferreira and Vane 1967). That some catecholamine removal occurs in the lesser circulation as well has also been previously observed, in experimental animals (Elliott 1905, Eisenmann *et al.* 1964, Hughes *et al.* 1969, Glinn and 1972) as well as in man (Blum *et al.* 1969, Gillis *et al.* 1972).

the forearm the removal of  $^3\text{H}$ -NA was nearly twice as great as that of  $^{14}\text{C}$ -inulin, suggesting the presence of specific trapping mechanisms for  $^3\text{H}$ -NA in these tissues; however, the present results give no indication as to the identity or cellular localization of such systems (*cf.* Iversen 1971, Glinn and Vane 1969, Gryglewski and Vane 1970, Oswald Branco 1973).

the lesser circulation the removal of  $^3\text{H}$  NA was far less prompt and efficient than that in a systemic bed, and about equal to that of  $^{14}\text{C}$ -inulin, in the first two sampling periods. suggests that  $^3\text{H}$  NA was initially removed from the pulmonary blood stream by unspecific diffusion/filtration.

the present results give no clue as to the ultimate fate of the NA removed from the pulmonary circulation, in man. But results obtained in studies of isolated perfused rat or rabbit lungs indicate that the NA removed from the pulmonary vascular bed in these species is very likely taken up into sites in which it is metabolized rather than stored, histochemical studies appear to implicate capillary endothelial cells as the probable cellular sites of uptake (Hughes *et al.* 1969, Alabaster and Bakhle 1973, Iwasawa *et al.* 1973, Iwasawa and Gillis 1974).

That considerable transcapillary exchange of water does occur even in the pulmonary circulation has been demonstrated in experiments with isolated perfused lungs from various species (Guyton and Lindsey 1959, Hango *et al.* 1966) as well as *in vivo* in anesthetized dogs (Warnd and Enns 1954) or unanesthetized sheep (Staub 1971). In the present study in unanesthetized man, a considerable and statistically highly significant fall in the  $^{14}\text{C}$ -inulin/ $^3\text{H}$ -albumin ratio was observed after one passage through the lungs, although less marked in the forearm. This finding is apparently in variance with the observation of Chabard and Enns (1954) in anesthetized dogs, that the concentration relationship for inulin was identical with that of the dye T 1824 used as a marker for plasma protein, after simultaneous injection of the two compounds into the

jugular vein, and sampling after passage through the lungs. It is at present not possible to decide on the reason for the discrepancy which may be due to species differences, to anaesthesia or to methodology. However that may be, the present findings strongly suggest that considerable transcapillary exchange of diffusible/filterable solutes does occur in the pulmonary capillaries of unanesthetized man. This may well be the immediate mechanism of removal from the lung capillary lumen not only of NA, but also of other circulating ergone of small molecular size. The ultimate fate of the particular ergone would depend on whether or not the extra-luminal tissues, including the capillary endothelium, contain specific mechanisms for concentrating and/or inactivating each of these compounds.

This research was supported by a grant (to L. S., G. B. and L. K.) from the Swedish Medical Research Council (K72 19X 3783-01) and by Pulmonary SCOR Grant HL 15063, NHLI, USPHS (to A. M.). The skilful technical assistance of Miss B. Carlgren, Mrs M. Arvidsson, Mrs I. M. Eriksson and Ms L. Karlsson is gratefully acknowledged.

## References

- ALABASTER, V. A. and Y. S. BAKHLE, The removal of noradrenaline in the pulmonary circulation of the isolated lung. *Brit. J. Pharmacol.* 1973 47 325-331.
- BIRCH, P. J. C., BOILEAU, L. and CAMPEAU, D., Norepinephrine inactivation in the pulmonary circulation of animals and humans (abstr.). *Clin. Res.* 1969 17 230.
- BURN, J. H., The action of tyramine and ephedrine. *J. Pharmacol. exp. Ther.* 1932 46 75-95.
- CELANDER, O. and S. MELLANDER, Elimination of adrenaline and noradrenaline from the circulating blood. *Nature (Lond.)* 1955 176 973-974.
- CHENARD, F. P. and T. ENNS, Transcapillary pulmonary exchange of water in the dog. *Amer. J. Physiol.* 1954 178 197-202.
- COLE, D. R., K. E. COOPER, R. F. MOTTRAM and J. V. OCCLESIAHAW, The removal of blood samples withdrawn from deep forearm veins via catheters passed upstream from the median cubital vein. *J. Physiol. (Lond.)* 1958 142 323-328.
- EREMAN, B., L. BRYANT and T. WALTUCH, Metabolism of vasomotor agents by the isolated perfused lung. *J. thorac. cardiovasc. Surg.* 1964 48 798-806.
- ELLIOTT, T. R., The action of adrenalin. *J. Physiol. (Lond.)* 1903 32 401-467.
- FERRERA, S. H. and J. R. VANE, Half-lives of peptides and amines in the circulation. *Nature (Lond.)* 1965 215 1237-1240.
- GILLIS, C. N., N. M. GREENE, L. H. CROONAU and G. L. HAMMOND, Pulmonary extraction of 5-hydroxytryptamine and norepinephrine before and after cardiopulmonary bypass in man. *Circulat. Res.* 1972 30 666-674.
- GDON, R. and J. R. VANE, Disappearance of catecholamines from the circulation. (*Nature (Lond.)* 1964 219 740).
- GRYGLEWSKI, R. and J. R. VANE, The inactivation of noradrenaline and isoprenaline in dogs. *Brit. J. Pharmacol.* 1970 39 573-584.
- GUYTON, A. C. and A. W. LINDSAY, Effect of elevated left atrial pressure and decreased plasma protein concentration on the development of pulmonary edema. *Circulat. Res.* 1959 7 649-657.
- HAUCK, A., P. K. M. LUNDE and B. A. WAALER, Transvascular fluid balance in the lung. *J. Physiol. (Lond.)* 1966 186 94-95P.
- HUGHES, J., C. N. GILLIS and F. E. BLOOM, Uptake and disposition of norepinephrine in perfused rat lung. *J. Pharmacol. exp. Ther.* 1969 169 237-243.
- IWASAWA, Y. and C. N. GILLIS, Pharmacological analysis of norepinephrine and 5-hydroxytryptamine removal from the pulmonary circulation. Differentiation of uptake sites for each amine. *J. Pharmacol. exp. Ther.* 1974 188 386-393.
- IWASAWA, Y., C. N. GILLIS and G. AGHAZANIAN, Hypothermic inhibition of 5-hydroxytryptamine and norepinephrine uptake by lung: Cellular location of amines after uptake. *J. Pharmacol. exp. Ther.* 1977 186 498-507.

- VERLEY, L. L., Role of transmitter uptake mechanisms in synaptic neurotransmission. *Brit. J. Pharmacol.* 1971 41 571-591.
- WATKINS, A. A., B. R. LEVINE and M. J. ANTONUCCI, Uptake of catecholamines in guinea-pig lung: influence of carazolol and asphyxia. *J. Allergy clin. Immun.* 1975 55 170-181.
- ZERWALD, W. and D. BRAMCO, The effects of drugs and denervation on removal and accumulation of  $^{125}$ I noradrenaline in the perfused hindlimb of the dog. *Namyo-Schmidberg Arch. Pharmacol.* 1973 277 175-190.
- HYATT, N. C., Steady state pulmonary transvascular water filtration in unanesthetized sheep. *Suppl. to Circulat. Res.* 1971 28-29 135-139.
- WILLIAMS, L., Studies of noradrenaline biosynthesis in nerve trunks. *Acta physiol. scand.* 1966 87 441-454.



## Rat Liver Mitochondrial Enzyme Activities in Hypoxia

By

VUOKKO L. KINNULA

Received 24 February 1975

---

### Abstract

KINNULA, V. L. *Rat liver mitochondrial enzyme activities in hypoxia.* Acta physiol. scand. 1975. 95. 54-59

Rat liver mitochondrial enzyme activities were measured after exposing the animals to the atmospheric pressure of 380 mm Hg for 5 h and 14 days. Succinate dehydrogenase and succinate oxidase activities increased significantly during the hypoxic period of 14 days. No change was observed in cytochrome oxidase activity. Malate dehydrogenase and glutamate dehydrogenase activities increased somewhat, but not significantly. The efficiency of oxidative phosphorylation (the ADP/O ratio) in the isolated mitochondria remained unchanged. The exact mitochondrial protein values showed a 15% decrease as compared with the control group. The concentrations of cytochromes did not change significantly in the hypoxic group. During the short hypoxic period succinate dehydrogenase, succinate oxidase and cytochrome oxidase activities increased as compared with those in the control group.

**Key words.** liver hypoxia, mitochondria, cytochromes, oxidative phosphorylation, succinate dehydrogenase

---

Mitochondria are resistant to low oxygen tension at the tissue level (Chance 1965). They can be synthesized in yeast in anaerobic conditions though these mitochondria are not functionally normal (Criddle and Schatz 1969). Low oxygen tension in the inspired air, however, may result in damage in mitochondria, as can be observed in electron microscope studies (Glinzmann and Ericsson 1966) or radioisotope experiments (Rabinowitz *et al.* 1971).

As a result of adaptation to low oxygen tension the oxygen-carrying capacity of the blood increases and the oxygen level in the various tissues remains relatively high. During moderate hypoxia mitochondria are capable of exhibiting normal functional metabolism (Park *et al.* 1973) and it seems probable that no damage is done to them (Sanders *et al.* 1965). It is evident that mitochondrial metabolism can adapt to these circumstances. The type and significance of this adaptation has not been resolved. In addition there is much controversy over mitochondrial function in moderate hypoxia and during the adaptation process. In the present study mitochondrial enzyme activities have been investigated in 2 hypoxic periods of different length. Part of this investigation has been communicated in a preliminary form (Kinnula 1974).

## Material and Methods

Star rats of both sexes (age about 4 months, weight 250-330 g) were used as experimental animals, pups as produced in the aid of hypobaric pressure chamber. Care was taken that in the same experiment the same sex and age ratios were achieved in both the control and hypoxic groups. The hypoxic rat was exposed to an atmospheric pressure of 380 mm Hg corresponding to an altitude of about 5500 m. Low pressure as obtained in the chamber in about 10 min. Rats are maintained in the chamber for 14 days in the acute experiments and for 14 days in the chronic experiments. The chamber was opened every 3 days for few minutes to weigh, feed and water the animals. Rats were given food (commercial standard rat pellets) and water *ad lib.* throughout the experiments.

After the hypoxic periods the rats were decapitated and blood samples were taken for hemoglobin and hematocrit determinations. The livers were rapidly removed and placed in 0.32 M sucrose-2 mM Tris buffer (pH 7.4) at 0°C. The livers were homogenized with a glass homogenizer fitted with motor-driven flat pestle. A sample from the total homogenate was taken for enzyme and cytochrome assays, determinations. The mitochondrial fraction was isolated according to O'Brien and Kalf (1967). One washing of the acetone was carried out at 5200 g for 10 min. The mitochondrial pellet was used immediately. Mitochondrial phosphorylation (ADP/ATP ratio) was determined polarographically by means of Clark type oxygen electrode using succinate as substrate by the modified method of Hansson (1967).

Succinate dehydrogenase (EC 1.3.99.1) was determined spectrophotometrically by 2 methods from the total homogenate and from the isolated mitochondria. In the first method potassium ferricyanide is used as an artificial electron acceptor and the determination was performed by the method of Botter 1955). The dye concentration was checked in the laboratory so as to obtain the fastest reaction velocity possible. The specific activity is expressed as  $\mu\text{mol/min/mg protein}$  as calculated from the absorbency change at 400 nm. In the second method dichlorotetraphenol was used as an artificial electron acceptor after Jones *et al.* (1955). The specific activity is expressed as  $\mu\text{mol/min/mg protein}$ .

Succinate oxidase and cytochrome oxidase (EC 1.3.3.1) activities were determined manometrically (Unluvar 1964). The oxygen uptake at 37°C was measured for 60 min after the initial equilibration of 5 min. The enzyme activities are expressed as  $\mu\text{l O}_2/\text{min/mg protein}$ .

Malate dehydrogenase (EC 1.1.1.37) and glutamate dehydrogenase (EC 1.4.1.3) activities were determined by measuring the rate of  $\text{NAD}^+$  reduction by the methods of Siegel and Englund (1961) and Bensley *et al.* (1959). The activities are expressed as  $\mu\text{mol NAD}^+$  reduced/min/mg protein.

The cytochrome assays were performed with double beam spectrophotometer (Perkin Elmer Double Beam 154). The reduction of cytochromes was accomplished in the presence of cytochrome and nicotinamide A by the method of Chance and Williams (1955), as modified by Hallstrom *et al.* (1972). The calculations were performed according to Vannote (1966). The cytochrome concentrations are expressed as  $\mu\text{moles/mg protein}$ .

The exact mitochondrial protein values were calculated from the cytochrome  $\text{aa}_3$  concentrations in the total homogenate and in the isolated mitochondria. Since cytochrome  $\text{aa}_3$  is a specific mitochondrial component, assays of the cytochrome can be used to evaluate the amount of mitochondria in tissues (Schollmeyer and Klingenberg 1962).

Glucose-6-phosphatase (EC 3.1.3.9), the marker enzyme of the microsomal fraction, was determined according to Leighton *et al.* (1968) with slight modification. The inorganic phosphate released during the incubation period (30 min, 37°C) was determined according to Erster *et al.* (1950).

Acid phosphatase (EC 5.1.3.2), the marker enzyme of the lysosomal fraction, was determined by the modified method of Applebaum *et al.* (1955). The inorganic phosphate was determined as mentioned above. The activity is expressed as the amount of inorganic phosphate released.

The protein concentrations were determined by the method of Lowry *et al.* (1951).

## Results

**Chronic hypoxia.** Although the body weight of the hypoxic animals was lower than that of the controls no changes were observed in liver weight to body weight ratios. Hemoglobin and hematocrit values increased about 50% during this period (Table I).

TABLE I Effect of chronic hypoxia on body weights, liver weight to body weight ratios, hemoglobin concentrations and hematocrit values.

	Control	Hypoxic (14 days, 380 mm Hg)
Body weight change (%)	+12.9 ± 0.80 (15)	-3.6 ± 0.71 (15)
Liver wt. g/100 g body wt.	3.70 ± 0.80 (15)	3.70 ± 0.39 (15)
Hemoglobin, g/l	145.3 ± 6.2 (7)	210.7 ± 4.0 <sup>b</sup> (7)
Hematocrit	0.43 ± 0.02 (7)	0.66 ± 0.02 <sup>b</sup> (7)

Values are means ± S.D. with the number of experiments in parenthesis. - the lowest weight (14) was observed on the 5th day in hypoxia. <sup>b</sup> =  $p < 0.001$  -  $p < 0.001$  (Student's test).

The ADP/O ratio did not change during the chronic hypoxia, and as far as the cytochrome concentrations were concerned cytochromes *a<sub>s</sub>* and *b* were slightly lower but the difference between the control and hypoxic groups was not significant (Table II). The mitochondrial protein values, as calculated from the cytochrome *a<sub>s</sub>* concentrations, showed a decrease in the hypoxic group as compared with the control values (-15%).

In the mitochondrial enzyme activities, the chronic hypoxic period (2 weeks) resulted in an increase in succinate oxidase and succinate dehydrogenase activities. Cytochrome oxidase activity remained unchanged. Malate dehydrogenase and glutamate dehydrogenase activities increased somewhat, but not significantly. These results are shown in Table III. When the control value in each experiment was marked as 100, succinate dehydrogenase activity in the hypoxic group increased by 31% in the total homogenate and by 23% in isolated mitochondria (K<sub>2</sub>Fe(CN)<sub>6</sub> as an electron acceptor). The corresponding values for the dichloroindophenol method were 24% and 20%. The increase in succinate oxidase activity in the hypoxic group was 23%.

**Acute hypoxia** During the hypoxic period of 5 hours succinate dehydrogenase, succinate oxidase and cytochrome oxidase activities increased (Table IV). Succinate oxidase activity in isolated mitochondria increased by 35% and cytochrome oxidase by 21%.

The lysosomal contamination was 15% and the microsomal contamination 3.5% in mitochondria. It was observed that more pure mitochondrial fraction could be obtained by washing several times with the isolation medium. However the isolation of this fraction takes time and may damage the functional integrity of the mitochondria.

TABLE II Comparison of mitochondrial cytochrome concentrations, ADP/O ratios and protein values in altitude-exposed and control rats after 2 weeks.

	Control	Hypoxic
Cyt <i>a<sub>s</sub></i>	0.363 ± 0.101 (8)	0.330 ± 0.081 (8)
Cyt <i>b</i>	0.119 ± 0.026 (8)	0.116 ± 0.026 (8)
Cyt <i>c</i>	0.325 ± 0.064 (8)	0.314 ± 0.052 (8)
Cyt <i>c<sub>1</sub></i>	0.172 ± 0.067 (8)	0.146 ± 0.061 (8)
ADP/O	1.89 ± 0.21 (3)	1.97 ± 0.17 (3)
Protein	53.1 ± 15.8 (3)	45.2 ± 17.8 (3)

Cytochrome concentrations are expressed as nmol/mg protein and protein values mg/g wet weight as calculated from cytochrome *a<sub>s</sub>* concentrations in the total homogenate and in isolated mitochondria. Values are means ± S.D. with the number of experiments in parenthesis.

3. III. Effect of chronic hypoxia (2 weeks) on succinate oxidase (SUCC OX), succinate dehydrogenase (SDH), cytochrome oxidase (CYT OX), malate dehydrogenase (MDH) and glutamate dehydrogenase (GDH) activities.

Enzyme	Fraction	Control	Hypoxic
SDH	Total homogenate	0.043 ± 0.006 (7)	0.057 ± 0.011 (7) <sup>a</sup>
CYT OX	Total homogenate	4.77 ± 1.01 (6)	6.05 ± 2.53 (6)
CYT OX	Mitochondria	5.23 ± 0.92 (6)	6.35 ± 1.13 (6) <sup>b</sup>
MDH	Mitochondria	18.4 ± 4.67 (5)	18.8 ± 4.75 (5)
GDH	Mitochondria	2 617 ± 557 (8)	2 941 ± 544 (8)
GDH	Mitochondria	122.6 ± 33.6 (7)	134.3 ± 42.2 (7)

Activities are expressed as  $\mu\text{mol O}_2/\text{min}/\text{mg}$  protein for cytochrome oxidase and succinate oxidase, units/mg protein for succinate dehydrogenase ( $\text{K}_3\text{Fe}(\text{CN})_6$  as an electron acceptor),  $\mu\text{mol}/\text{min}/\text{mg}$  (diapirone) as an electron acceptor,  $\mu\text{mol}/\text{min}/\text{mg}$  protein for malate dehydrogenase and glutamate dehydrogenase. Values are means  $\pm$  S.D. with the number of experiments in parenthesis. <sup>a</sup>  $p < 0.02$ , <sup>b</sup>  $p < 0.1$  (Student's *t*-test).

### Discussion

Acclimatization of the animals to low oxygen pressure for 2 weeks was indicated by the changes in blood and body weight. No damage was caused to the mitochondria in these experiments, as the efficiency of oxidative phosphorylation did not change. These results agree with previous studies (Strickland *et al.* 1962, Ziegler 1967, Gold *et al.* 1973). All these observations point to the fact that mitochondria are very resistant during altitude stress. Slight uncoupling of oxidation and phosphorylation has been observed, however after 4 days of exposure to 7 520 m (Nelson *et al.* 1967). It is probable that this kind of phenomenon is a transient change which is associated with the onset of altered conditions and which returns to normal once the adaptation process proves possible.

As far as the mitochondrial enzyme activities at altitudes are concerned the results of this study show an increase in succinate dehydrogenase activities in both the acute and chronic experiments. This reflects the dependence of the increase in activity on some kind of activation and not on an increase in enzyme protein. It has been concluded that the increase in succinate dehydrogenase activity is the result of a structural change in an enzyme protein (Aitch and Ramanarma 1969). In this study cytochrome oxidase activity did not

TABLE IV. Effect of acute hypoxia (5 h) on succinate oxidase (SUCC OX), cytochrome oxidase (CYT OX), succinate dehydrogenase (SDH), malate dehydrogenase (MDH) and glutamate dehydrogenase (GDH) activities in isolated mitochondria.

Enzyme	Control	Hypoxic
SDH	0.224 ± 0.045 (8)	0.288 ± 0.048 (8) <sup>a</sup>
SUCC OX	4.47 ± 1.00 (3)	6.05 ± 0.91 (3) <sup>b</sup>
CYT OX	17.3 ± 4.23 (3)	20.9 ± 4.13 (3) <sup>b</sup>
MDH	1 771 ± 284 (5)	1 943 ± 499 (5)
GDH	79.9 ± 43.3 (5)	81.4 ± 38.1 (5)

The activities are expressed as  $\mu\text{mol O}_2/\text{min}/\text{mg}$  protein for cytochrome oxidase and succinate oxidase, units/mg protein for succinate dehydrogenase ( $\text{K}_3\text{Fe}(\text{CN})_6$  as an electron acceptor) and  $\mu\text{mol}/\text{min}/\text{mg}$  protein for malate dehydrogenase and glutamate dehydrogenase. Values are means  $\pm$  S.D. with the number of experiments in parenthesis. <sup>a</sup>  $p < 0.05$ , <sup>b</sup>  $p < 0.01$ , <sup>c</sup>  $p < 0.1$  (Student's *t*-test).

change during the longer hypoxic period, though it increased during the acute experiment. These results point to permanent activation of the succinate dehydrogenating enzyme as a consequence of hypoxic stress and adaptation. The transient increase in cytochrome c oxidase activity may be part of the hypoxic reaction.

The exposure of rats to low atmospheric pressure for two weeks resulted in a decline of the cytochromes. This change was, however, small and not statistically significant. The relation of the cytochromes to hypoxia has not been resolved. In yeast cells it has been noted that oxygen deficiency results in a decrease in cytochromes (Biggs and Linnane 1969). Hallman (1971) has observed that the concentration of cytochromes  $c + c_1$  does not increase normally when the environment of neonatal rats becomes hypoxic. On the other hand it has been concluded that moderate hypoxia may even cause an increase in cytochrome concentrations (Sherzer and Cascarano 1972). More studies especially in more severe hypoxia are needed. The corresponding phenomena of the heart muscle might be of special interest because of the importance of oxidative metabolism.

It may be concluded that moderate hypoxia leads to some change in the mitochondrial metabolism, but does not destroy the mitochondria. The efficiency of oxidative phosphorylation in altitude-exposed rats remains the same as in the controls. The low mitochondrial protein values indicate that the mitochondrial population has decreased or the composition of the mitochondria has changed. As to the increased enzyme activities in the hypoxic group their significance remains unclear. It is possible that this increased enzyme function in moderate hypoxia is an attempt to adapt the tissue to increased energy demands.

## References

- ATHAL, H. N. and T. RAMASARMA, Activation of liver succinate dehydrogenase in rats exposed to baric conditions. *Biochem. J.* 1969 115 77-83.
- APPELMAIR, F. R., WATTIAUX and C. deDUVE, Tissue fractionation studies. 5. The association of phosphatase with a special class of cytoplasmic granules - rat liver. *Biochem. J.* 1955 59 438-445.
- BEAUFAY, Y. H. D. S., BENDALL, P., BAUDHUIN and C. deDUVE, Tissue fractionation studies. 12. Intracellular distribution of some dehydrogenases, alkaline deoxyribonuclease and iron in rat-liver tissue. *Biochem. J.* 1959 73 623-628.
- BIGGS, W. R. and A. W. LINNANE, The effect of oxygen on the composition and organization of the iron transport system of yeast. *Biochim. biophys. Acta (Amst.)* 1963 73 785-788.
- BONNER, W. H. Succinic dehydrogenase. In *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan). Academic Press, New York and London, 1955 713-729.
- CHANCE, B. and G. R. WILLIAMS, Respiratory enzymes - oxidative phosphorylation II. Difference spectra. *J. Biol. Chem.* 1955 217 395-407.
- CHANCE, B., Reaction of oxygen with the respiratory chain - cells and tissues. *J. gen. Physiol.* 1963 163-195.
- CRIDDLE, R. S. and G. SCHATZ, Promitochondria of anaerobically grown yeast. I. Isolation and biochemical properties. *Biochemistry* 1969 8 322-334.
- ERNSTER, L., R. ZETTERSTRÖM and O. LINDBERG, A method for the determination of tracer phosphorus in biological material. *Acta chem. scand.* 1950 4 941-947.
- GLICKSMANN, W. H. and J. L. E. ERICSSON, Observations on the subcellular organization of hepatic parenchymal cells. II. Evolution of reversible alterations induced by hypoxia. *Lab. Invest.* 1966 15 76.
- GOLD, A. J., T. F. JOHNSON and L. C. COSTELLO, Effects of altitude stress on mitochondrial function. *Amer. J. Physiol.* 1973 224 946-949.
- GREEN, D. E., S. MI and P. M. KOSKOFF, Studies on the terminal electron transport system. I. Succinate dehydrogenase. *J. Biol. Chem.* 1955 217 551-567.

- LALLMAN, M., Changes in mitochondrial respiratory chain proteins during perinatal development. Evidence of the importance of environmental oxygen tension. *Biochem. biophys. Acta* (Amst.) 1971. 251. 360-372.
- LALLMAN, M., P. MÄRZELL and L. HANSEN, Levels of cytochromes in heart, liver, kidney and brain in the developing rat. *Experientia* (Basel) 1972. 28. 1408-1410.
- LAMARCA, I., Hydrogen transfer into mitochondria in the metabolism of ethanol. II. Effect of disulfiram on the hydrogen and energy transfer. *Ann. Med. exp. Fenn.* 1967. 45. 46-56.
- GREULA, V., Enzyme activities of liver mitochondria from altitude exposed rats. *Acta physiol. scand.* 1974. 91. 12A.
- JORDON, F. B., POOLE, H., BRADY, P., BAUDSON, J. F., CONVEY, E., FOWLER and C. DE DUVE, The large-scale separation of peroxisomes, mitochondria, and lysosomes from the livers of rats injected with Triton WR 1139. *J. cell. Biol.* 1968. 37. 482-513.
- LOWRY, O. H., H. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951. 193. 265-275.
- NELSON, R. D., B. HIGMAN and P. D. ALTLAND, Oxidative phosphorylation during altitude acclimation in rats. *Amer. J. Physiol.* 1967. 213. 1414-1418.
- O'BRIEN, F. W. and G. F. KALT, Ribosomes from rat liver mitochondria. I. Isolation procedures and contamination studies. *J. Biol. Chem.* 1967. 242. 2173-2179.
- PALE, C. D., L. MELA, R. WRATON, J. REILL, P. FISHER and E. ABERDEEN, Cardiac mitochondrial activity in acute and chronic cyanosis. *J. Surg. Res.* 1973. 14. 139-146.
- RABINOWITZ, M., V. ARSHANSKY, R. ALON, N. J. OROSH, R. ZAK and K. G. MAM, Synthesis and turnover of heart mitochondria in normal, hypertrophied and hypoxic rat. I. *Cardiac Hypertrophy* (Ed. N. Alpert). New York: Academic Press. 1971. 283-299.
- SAMUELS, A., P. D. M. HALL and A. T. MILLER, Jr., Some effects of hypoxia on respiratory metabolism and protein synthesis in rat tissues. *Amer. J. Physiol.* 1965. 209. 443-446.
- SCHOLLMEYER, P. and M. KLINGENSCH, Über den Cytochrom-Gehalt tierischer Gewebe. *Biochem. Z.* 1962. 335. 426-439.
- SPLETZER, H. G. and J. CASARANO, Mitochondrial alterations in heart, liver and kidney of altitude-acclimated rats. *Amer. J. Physiol.* 1972. 223. 632-636.
- SWOOL, L. and B. ENGLAND, Beef heart malic dehydrogenase. I. Properties of the enzyme purified from extracts of acetone-dried powders. *Biochem. biophys. Acta* (Amst.) 1961. 54. 67-76.
- STICKLAND, E. H., E. ACKERMAN and A. ANTHONY, Respiration and phosphorylation in liver and heart mitochondria from altitude-exposed rats. *J. appl. Physiol.* 1962. 17. 535-538.
- UNOWIT, W. W., R. H. BURKE and J. F. STAUFFER, *Automatic techniques*. Burger Publishing company 1964. 159-167.
- VANNITE, W. H., Molecular proportion of the fixed cytochrome components of the respiratory chain of Kalke-Hartree particles and beef heart mitochondria. *Biochem. biophys. Acta* (Amst.) 1966. 115. 175-178.
- ZWOLFE, F. D., Respiratory and phosphorylative responses to hypoxia and food restriction. *Amer. J. Physiol.* 1967. 212. 197-202.

change during the longer hypoxic period, though it increased during the acute exposure. These results point to permanent activation of the succinate dehydrogenating enzyme system as a consequence of hypoxic stress and adaptation. The transient increase in cytochrome c oxidase activity may be part of the hypoxic reaction.

The exposure of rats to low atmospheric pressure for two weeks resulted in a decline of the cytochromes. This change was, however, small and not statistically significant. The reaction of the cytochromes to hypoxia has not been resolved. In yeast cells it has been noted that oxygen deficiency results in a decrease in cytochromes (Biggs and Linnane 1964). Hallman (1971) has observed that the concentration of cytochromes c + c does not increase normally when the environment of neonatal rats becomes hypoxic. On the other hand it has been concluded that moderate hypoxia may even cause an increase in cytochrome concentrations (Sherzer and Cascarano 1972). More studies especially in more severe hypoxia are needed. The corresponding phenomena of the heart muscle might be of special interest because of the importance of oxidative metabolism.

It may be concluded that moderate hypoxia leads to some change in the mitochondrial metabolism, but does not destroy the mitochondria. The efficiency of oxidative phosphorylation in altitude-exposed rats remains the same as in the controls. The low mitochondrial protein values indicate that the mitochondrial population has decreased or the composition of the mitochondria has changed. As to the increased enzyme activities in the hypoxic group their significance remains unclear. It is possible that this increased enzyme function in moderate hypoxia is an attempt to adapt the tissue to increased energy demands.

## References

- ARTHUR, H. N. and T. RAMAKRISHNA, Activation of liver succinate dehydrogenase in rats exposed to hypobaric conditions. *Biochem. J.* 1969 115 77-83.
- APPELBAUM, F. R., WATTIAUX and C. deDUVE, Tissue fractionation studies. 5. The association of cytochrome phosphatase with a special class of cytoplasmic granules in rat liver. *Biochem. J.* 1955 59 438-443.
- BEAUFAY, Y. H. D. S., BENDALL, P. B., UDENFUT and C. deDUVE, Tissue fractionation studies. 12. Intracellular distribution of some dehydrogenases, alkaline deoxyribonuclease and iron in rat-liver tissue. *Biochem. J.* 1959 73 623-628.
- BIGGS, D. R. and A. W. LINNANE, The effect of oxygen on the composition and organization of the electron transport system of yeast. *Biochim. biophys. Acta* (Amst.) 1961 78 785-788.
- BOERNER, W. B. Succinic dehydrogenase. I. *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan). Academic Press, New York and London, 1955 723-729.
- CHANCE, B. and G. R. WILLIAMS, Respiratory enzymes in oxidative phosphorylation II. Differences between species. *J. Biol. Chem.* 1955 217 395-407.
- CHANCE, B., Reaction of oxygen with the respiratory chain in cells and tissues. *J. gen. Physiol.* 1963. 163-193.
- CRIDDLE, R. S. and G. SCHATZ, Promitochondria of anaerobically grown yeast. I. Isolation and biochemical properties. *Biochemistry* 1969 8 322-334.
- ERNSTER, L., R. ZITTELSTROM and O. LINDBERGH, A method for the determination of tracer phosphates in biological material. *Acta chem. scand.* 1950. 4 941-947.
- GLIDEMAN, W. H. and J. L. E. ERIKSSON, Observations on the subcellular organization of hepatic parenchymal cells. II. Evolution of reversible alterations induced by hypoxia. *Lab. Invest.* 1966. 15 762-770.
- GOLD, A., J. T. F. JOHNSON and L. C. COSTELLO, Effects of altitude stress on mitochondrial function in rat liver. *J. Physiol.* 1973 224 946-949.
- GREEN, D. E., S. M. and P. M. KOSKOFF, Studies on the terminal electron transport system. I. Succinate dehydrogenase. *J. Biol. Chem.* 1955. 217 551-567.

re (MAP), sampling of arterial blood and venous infusions respectively. Arterial  $P_{O_2}$ ,  $P_{CO_2}$ , and pH determined. Details of the procedures have been presented previously (Ahn 1975).  
 as an additional aim of the experiments was to determine the effect of topical application of 1 phane on ocular blood flow the animals were kept under pentobarbital anesthesia for varying times to blood flow determinations. Thus 3 monkeys were kept under anesthesia for 1 h, 3 for 4 h and 1 h. A thoracotomy was then performed and the radioactively labelled microspheres are injected into a ventricle of the heart. Ideally the spheres should act as non-recirculating blood flow indicator and be trapped within terminal arterioles, or capillaries, in proportion to blood flow. Microspheres of different sizes labelled with  $^{86}Sr$  and  $^{141}Ce$  respectively are used. According to the manufacturer (Company St Paul, Minnesota) the diameters of the spheres are  $15 \pm 5 \mu m$  and  $35 \pm 5 \mu m$  respectively and of 1 percent suspension of each sphere size were mixed and injected. The specific activity for both types was 10 mCi/g. At the start of the intracardiac injection and for the following 60 s blood was withdrawn from a cannulated femoral artery to serve as reference flow after which the animals were killed.

injections of KCl. Tissue samples were taken, blood and tissue samples were weighed and the activity was determined by gamma-spectrometry. Regional blood flow estimates could then be calculated for both sphere sizes by dividing the activity of the tissue sample by the activity per mg blood flow was calculated for the reference flow. Such determinations were made for lung, ocular tissues and various parts of the brain. Both lungs were removed, weighed and assayed and the radioactivity of 4 representative pieces was determined to estimate the overall leakage of the two sphere sizes through the systemic vascular system. Most of the pia was removed from the cerebellar hemispheres, and the radioactivity of the pia was measured, as rule as double samples. Double samples were taken from grey and white matter of the tail, temporal and occipital lobes. 4 samples were taken from the corpus callosum, and the choroid plexus was taken as one sample. Values obtained for ocular blood flow will be presented elsewhere.

## Results

the time of injection of the microspheres the following data were obtained, mean  $\pm$  S.E.

7). MAP  $121 \pm 7$  mm Hg, arterial  $P_{O_2}$ ,  $P_{CO_2}$ , and pH  $78 \pm 5$  mmHg,  $33.0 \pm 2.0$  mmHg and  $7.53 \pm 0.03$  units respectively. Blood flow to the lung was  $29 \pm 9$  g/min per 100 g tissue isolated for the  $15 \mu m$  spheres and  $32 \pm 9$  for the  $35 \mu m$  spheres.

Table I presents the blood flow values obtained with the two sphere sizes in various parts of the brain. Flow values for grey matter are higher with  $35 \mu m$  spheres than with  $15 \mu m$  spheres while the reverse happens for white matter including corpus callosum. No difference is seen for the choroid plexus. With  $15 \mu m$  spheres there were no significant differences in flow through the various lobes for either grey or white matter.

For both sphere sizes a certain amount of spheres were trapped in extracerebral pial arterioles, and thus erroneously excluded in rCBF-determinations. The number of spheres trapped in pial arterioles corresponds to a blood flow of  $37 \pm 7$  ( $n=7$ ) and  $1756 \pm 486$  ( $n=7$ ) g/min per 100 g pia for  $15 \mu m$  and  $35 \mu m$  spheres respectively. Thus with  $35 \mu m$  spheres, on weight basis, almost 30 times as many spheres are trapped in pial arterioles as in grey matter while for  $15 \mu m$  spheres this ratio is about 1.

To evaluate the significance of the difference in flow obtained with the two sphere sizes in the various tissues, the mean value of the sample differences (flow calculated from  $35 \mu m$  spheres) - (flow calculated from  $15 \mu m$  spheres) is presented in Table II. The differences were significant for both grey and white matter including corpus callosum. A similar calculation for the pia also resulted in a significant difference,  $1756 \pm 411$  ( $n=11$ ) g/min per 100 g pia ( $p=0.005$ ).



## Radioactively Labelled Microspheres in Regional Cerebral Blood Flow Determinations

### A study on monkeys with 15 and 35 $\mu$ m spheres

By

ALBERT ALM

Received 3 March 1975

---

#### Abstract

ALM, A. Radioactively labelled microspheres in regional cerebral blood flow determinations. A study on monkeys with 15 and 35  $\mu$ m spheres. *Acta physiol scand.* 1975 95 60-65

15  $\mu$ m and 35  $\mu$ m radioactive microspheres, differently labelled were used simultaneously for 1 determinations in monkeys. Flow values calculated from data for 35  $\mu$ m spheres were  $27 \pm 7$  g/min 100 g higher for grey matter and  $10 \pm 3$  g/min per 100 g lower for white matter than flow values calculated from 15  $\mu$ m spheres, while both sphere sizes resulted in similar values for the choroid plexus. A number of 35  $\mu$ m spheres were trapped in extracerebral pial arterioles. It is concluded that entrance of relatively large arterioles of the 35  $\mu$ m spheres explains the differences in flow values, and that this level the use of 35  $\mu$ m spheres, and larger in rCBF-determinations.

---

The use of intracardiac injections of radioactively labelled microspheres has made it possible to make simultaneous determinations of blood flow through various parts of the (Rudolph and Heymann 1967). The method has been used for determinations of blood flow through whole organs and for determinations of regional blood flow within organs. However studies on cardiac muscle (Domenech *et al* 1969 Yipintsoi *et al* 1973 and *et al* 1974) and kidney cortex (Katz *et al* 1971) have shown that localization of spheres within an organ may not be determined only by regional blood flow. Other factors affect sphere distribution such as axial streaming of spheres, and spheres that either pass through the capillary bed or become trapped in arterioles proximal to the tissue studied. These factors are influenced by the size of the injected spheres. Thus in the present study spheres of two sizes were injected simultaneously to evaluate to what extent these factors influence regional cerebral blood flow (rCBF) determinations in monkeys.

#### Methods

7 monkeys of both sexes, 6 cynomolgus (*Macaca mrs*) and one vervet (*Cercopithecus thersites*), of between 2.0 and 3.3 kg were used. The animals were anesthetized, tracheotomized and artificially ventilated. Both femoral arteries and one femoral vein were cannulated for measurements of mean arterial blood pressure and for the injection of microspheres.

eres in this respect. Large spheres may become trapped in too large arterioles not included in the sample, and axial streaming may cause erroneous distribution of spheres within them. The degree of axial streaming increases with sphere size (Phillis and Dong 1971) and the error introduced in sphere distribution by this rheological property of the spheres may also be assumed to increase with sphere size. If these errors are of minor importance spheres of two sizes injected simultaneously should result in similar flow values. If not, it is likely that the larger spheres are in error provided that significant leakage of the smaller spheres has not taken place.

The difference between flow values calculated for 15 and 35  $\mu\text{m}$  spheres respectively in the present study (Table II) emphasizes that sphere size influences calculated rCBF-values. This difference can not be attributed to disturbances in local circulation caused by the microspheres since the two sphere sizes were mixed before injection. The assumption that the larger spheres are in error is supported by the high content of 35  $\mu\text{m}$  spheres within arterial vessels, which ideally should contain practically no spheres at all. Trapping of spheres within pial vessels can not be explained by axial streaming, and, with known data for vascular diameters within the brain, this suggests that trapping of 35  $\mu\text{m}$  spheres within large arterioles is the main obstacle that makes this sphere size, and larger unsuitable for rCBF-determinations. A similar error caused by large spheres in regional blood flow determinations has been reported by Katz *et al.* (1971) who found that 80  $\mu\text{m}$  spheres resulted in erroneously high blood flow values for the deeper layers of the kidney cortex due to entrapment in proximal parts of the arteriole. The number of 15  $\mu\text{m}$  spheres trapped within pial vessels in the present study shows that this source of error is unlikely to invalidate 15  $\mu\text{m}$  spheres, or smaller.

The present study permits no definite conclusions concerning the significance of a possible error due to axial streaming of 15  $\mu\text{m}$  spheres. Axial streaming in the large transcerebral arterioles may favour white matter on behalf of grey matter. In the present study the flow through grey matter is somewhat low. This may be due to the prolonged anesthesia. Barbiturate anesthesia reduces cortical blood flow (Landau *et al.* 1955). In previous reports on rCBF-studies on monkeys, anesthetized for a much shorter time, 15  $\mu\text{m}$  spheres resulted in mean values for white matter of 25–28 and for grey matter of 56 g/min per 100 g tissue (Ahn and Bull 1973, Ahn 1975). In the baboon James, Miller and Purves (1969) found flow values of 15–23 and 62–83 ml/min per 100 g tissue for white and grey matter respectively by intrarterial injections of  $^{133}\text{Xe}$ . The discrepancy between the results obtained by the two methods may have several explanations; difference in anesthetic depth, an inappropriate comparison since the anatomical localization of the flows measured by the two methods may not be identical, or axial streaming of the microspheres. That part of the discrepancy is explained by the latter possibility seems likely. However, studies on kidney cortex and cardiac muscle suggest that errors due to axial streaming of small spheres are not large enough to invalidate these in regional blood flow determinations. Thus in the dog kidney cortex 15  $\mu\text{m}$  spheres and radiolabelled frog red blood cells resulted in similar values for regional blood flow (Baehler *et al.* 1973). In cardiac muscle Yipintsoi *et al.* (1973) and Utley *et al.* (1974) found that larger spheres exaggerated flow through any area with more than average blood flow, usually subendocardial areas, as judged by smaller spheres.

TABLE I Calculated blood flow through various parts of the brain in g/min per 100 g tissue. Flow is calculated from 15  $\mu$ m spheres and from 35  $\mu$ m spheres are presented. Mean  $\pm$  S.E., 7 animals

Tissue	15 $\mu$ m spheres	35 $\mu$ m spheres
Grey matter		
Frontal lobe	35 $\pm$ 6	57 $\pm$ 10
Temporal lobe	28 $\pm$ 5	33 $\pm$ 6
Occipital lobe	40 $\pm$ 6	92 $\pm$ 19
White matter		
Frontal lobe	18 $\pm$ 2	11 $\pm$ 3
Temporal lobe	21 $\pm$ 3	8 $\pm$ 2
Occipital lobe	22 $\pm$ 2	15 $\pm$ 7
Corpus callosum	15 $\pm$ 2	7 $\pm$ 2
Choroid plexus	312 $\pm$ 44	316 $\pm$ 92

### Discussion

The pia vessels provide a vascular net supplying grey and white matter of the cerebral hemispheres through centripetal branches. There is no strict division into grey and white matter arterial vessels although a certain pattern exists. Thus superficial layers of grey matter are supplied by short cortical arterioles that do not enter white matter while deeper layers are supplied by cortical arterioles which sometimes also give branches to white matter. White matter is mainly supplied by larger transcerebral arterioles which, however, give branches also to deeper layers of grey matter (Campbell 1938, Saunders, Feindel and Carvalho 1966). The diameters of these vessels are of obvious importance for the choice of sphere size. Cortical arterioles may be as small as 15–20  $\mu$ m before entering brain substance (Nielsen and Owim 1967, Dahl 1973). The diameters of the larger transcerebral arterioles range from 30 to 50  $\mu$ m as they pass through grey matter (Saunders and Bell 1971). Since brain capillaries are in the order of 6 to 8  $\mu$ m passage of 15  $\mu$ m spheres through the cerebral vascular bed may be negligible. Spheres that pass through the systemic vascular bed are likely to become trapped within the lung. The similarity of the flow values calculated for the lung for 15 and 35  $\mu$ m spheres in the present study indicates that the difference between the two sphere sizes in this respect is not significant.

The use of microspheres in studies on regional blood flow implies that the spheres are trapped within small tissue samples in proportion to blood flow. Apart from passage of small spheres through the capillary bed two other possible errors might invalidate the results.

TABLE II Differences between blood flow values calculated from the two sphere sizes in various parts of the brain, and significance levels (Student's *t* test). The difference (flow calculated from 35  $\mu$ m spheres) – (flow calculated from 15  $\mu$ m spheres) was used. Mean  $\pm$  S.E. (n) = number of samples.

Tissue	Difference (g/min per 100 g tissue)	Significance level
Grey matter	27 $\pm$ 7 (42)	$p < 0.001$
White matter	-10 $\pm$ 3 (42)	$p < 0.005$
Corpus callosum	-8 $\pm$ 2 (28)	$p < 0.005$

- ARM, A. M. and M. A. HERRMANN, The circulation in the fetus in utero. Methods for studying distribution of blood flow, cardiac output and organ blood flow. *Circulat. Res.* 1967. 21: 163-184.
- DETS, R. L. de C. H. and M. A. BELL, X-ray microscopy and histochemistry of the human cerebral blood vessels. *J. Neurosurg.* 1971. 35: 128-140.
- DETS, R. L. de C. H. W. H. FENDEL and V. R. CAR ALMO, X-ray microscopy of the blood vessels of the human brain. *Med. Biol. Microsc.* 1965. 13: 108-122.
- III J. E. L. CARLSON, J. L. E. HOFFMAN, H. M. MARTINEZ and G. D. BRUCKBERG, Total and regional occluded blood flow measurements with 25  $\mu$ m, 13  $\mu$ m, 9  $\mu$ m, and filtered 1-10  $\mu$ m diameter microspheres and antipyrine in dogs and sheep. *Circulat. Res.* 1974. 35: 391-405.
- FEROL, T. W. A. DOWN, JR, P. D. SCANLON, T. J. KNAPP and J. B. BARNETT-WALSH, Regional distribution of diffusible tracers and carbonized microspheres in the left ventricle of isolated dogs heart. *Circulat. Res.* 1973. 33: 573-587.

and by diffusable tracers such as antipyrine. This unfavourable comparison was found between 15  $\mu\text{m}$  and 9  $\mu\text{m}$  spheres, but the difference was usually less than 10 per cent (Uhl *et al* 1974). Limitations of antipyrine in regional blood flow determinations, discussed by these latter investigators, led them to conclude that 9  $\mu\text{m}$  spheres are probably the best indicator for regional blood flow determinations in cardiac muscle.

The microspheres offer obvious advantages in rCBF-determinations. The possibility of an exact anatomical localization with high spatial resolution, without intervening surgery or trauma prior to flow determinations, may also be obtained with a diffusable tracer such as  $^{14}\text{C}$ -ethanol (Eklöf *et al* 1974). However unlike diffusable tracers, differently labelled microspheres permit repeated flow determinations, e.g. before and after a change in a physiological parameter. Thus radioactively labelled microspheres may be a valuable method for rCBF-determinations, but more information concerning the significance of axial streaming in cerebral vessels is needed. A comparison between flow values obtained with simultaneously injected 9  $\mu\text{m}$  and 15  $\mu\text{m}$  spheres may provide such information.

The author thanks professor Anders Bill for valuable discussions during the experiments and the preparation of the manuscript, and Miss Monica Thoren and Mrs Anita Östberg for valuable technical assistance.

This investigation was supported by a grant EY 00475 from the National Eye Institute, Public Health Service, USA and by a grant B74-14X 147 from the Swedish Medical Research Council.

## References

- ALM, A. The effect of stimulation of the cervical sympathetic chain on regional cerebral blood flow in monkeys. A study with radioactively labelled microspheres. *Acta physiol scand* 1975, 93, 483-489.
- ALM, A. and A. BILL. Ocular and optic nerve blood flow at normal and increased intraocular pressure in monkeys (*Macaca irus*)—study with radioactively labelled microspheres including flow determinations in brain and some other tissues. *Exp Eye Res* 1973, 15, 15-29.
- BAHLER, R. W. A. J. CATANZARO, J. H. STEIN and W. HUNTER. The radiolabelled frog red blood cell: A new marker of cortical blood flow distribution in the kidney of the dog. *Circulat Res* 1973, 32, 718-721.
- CAMPBELL, C. P. The vascular architecture of the cat's brain. A study by vital injection. *Assoc. Res. Dis. Proc* 1938, 18, 69-93.
- DAHL, E. The innervation of the cerebral arteries. *J Amer* 1973, 115, 53-63.
- DOMENECH, R. J. J. E. HOFFMAN, M. J. M. NOBLE, K. B. SAUNDERS, J. R. HENDON and S. SUMIDA. Total and regional coronary blood flow measured by radioactive microspheres—conscious and anesthetized dogs. *Circulat Res* 1969, 25, 581-596.
- EKLÖF, B., N. A. LARSEN, L. NELSON, K. NORRBERG, B. K. SUNDÖ and P. TÖRÖLÖF. Regional cerebral blood flow in the rat measured by the tissue sampling technique—critical evaluation using four indicators:  $^{14}\text{C}$ -antipyrine,  $^{14}\text{C}$ -ethanol,  $^3\text{H}$ -water and Xenon. *Acta physiol scand* 1974, 91, 1-10.
- JAMES, L. M., R. A. MILLAR and M. J. PURVES. Observations on the extrinsic neural control of cerebral blood flow in the baboon. *Circulat Res* 1969, 25, 77-93.
- KATZ, M. A., R. C. BLANTZ, F. C. RECTOR, J. and H. W. SELDIN. Measurement of intrarenal blood flow. I. Analysis of microsphere method. *Amer J Physiol* 1971, 220, 1903-1913.
- LANDAU, W. M., W. H. FREYGANG, J. L. P. ROLAND, L. SOKOLOFF and S. S. KETY. The local circulation of the living brain: values in the nonanesthetized and anesthetized cat. *Trans. Amer. neural Ass.* 1950, 80, 125-129.
- NELSEN, K. C. and C. OWMAN. Adrenergic innervation of pial arteries related to the circle of Willis in cat. *Brain Res* 1967, 6, 773-776.
- PIRBS, R. H. and L. DOWD. Nonuniform distribution of microspheres in blood flowing through a mesenteric artery. *Can. J Physiol Pharm.* 1970, 48, 415-421.

terase since a normal end-plate can be formed on an area of muscle that was never innervated previously (Hoffman 1951). The appearance of these receptors is therefore directly dependent on the presence of the motor nerve. Once formed, however, junctional receptors are kinetically stable: they persist for some time following denervation. Hartzell and Farnough (1972) noted approximately normal junctional receptor density after 12 weeks of denervation. Similarly Lomo and Rosenthal (1972) reported persistent, normal peaks of high ACh sensitivity at denervated end-plates when muscles were directly stimulated for 2 weeks. On muscle fibres innervated by a foreign nerve at a different site some of the old denervated end-plates remain sensitive to ACh for over a year (Frank *et al.* 1975).

These observations suggest that the long-term integrity of an end-plate, once established, might therefore be maintained by the metabolism of a normally active muscle rather than through some "trophic" influence of the nerve. In an attempt to discriminate among the various possibilities we studied the stability of junctional receptors at denervated end-plates on muscles in three different experimental situations: denervated muscles, denervated muscles kept active by direct electrical stimulation, and muscles innervated by a foreign nerve at some distance from the old denervated end-plates. The number of receptors was assessed by measuring the amount of [ $^{125}$ I]-bungarotoxin bound to old end-plates. This toxin binds specifically and nearly irreversibly to ACh receptors (Miledi and Potter 1971, Berg *et al.* 1972) and the high specific radioactivity of the toxin used in these experiments enabled us to quantitatively measure the ACh receptors at individual end-plates.

## Methods

### 1. Experimental preparations

The end-plates studied in these experiments were on soleus muscle fibres from 200–300 g Wistar albino male rats. Operations were performed under Nembutal anaesthesia. For each animal, one leg was operated and the other was used as control. 3 types of experimental material were prepared.

**Denervated-stimulated muscle.** A pair of stimulating electrodes was implanted in the adjacent musculature so that they were not in direct contact with the soleus muscle. Current between the two electrodes runs transverse to the muscle. The electrodes were platinum sheets covered with sponges Salicic (738 TRV Dove Corning) which was scraped away to expose a few mm<sup>2</sup> of metal on the side facing the soleus muscle. Solder connections were covered with Salicic. Stimulation began the same day as denervation (5–7 mm resection of the sciatic nerve at the popliteal fossa) and consisted of trains of 100 pulses of 3 ms duration at 10 Hz repeated every 12 s. The stimulus polarity was reversed between each train, and intensity was adjusted to give an easily visible and palpable contraction of the leg musculature. Effectiveness of the stimulation was checked daily and was confirmed at the end of the experiment by the absence of appreciable  $\alpha$ -bungarotoxin binding to extra-junctional membrane (see below). Absence of reinnervation was also confirmed just before fixation in the toxin by stimulation of the distal end of the cut sciatic nerve as it entered the muscle.

**Denervated muscle.** The muscle was denervated as described above. Stimulation was begun 10–14 days before the muscle was examined in order to reduce ACh sensitivity and toxin binding outside the end-plate region. In experiments lasting less than 3 weeks, no electrical stimulation was used. Absence of reinnervation was confirmed in each case.

**Cross-innervated muscles.** Soleus muscles were cross-innervated with the superficial branch of the fibular nerve. The branch was dissected, cut distally and transplanted onto the proximal dorsal surface of the soleus muscle and held in place with fibrin clot. About two weeks later the tibial nerve (which contains the soleus motor axons) was cut in the popliteal fossa and implanted into adjacent biceps muscle to prevent reinnervation of the soleus muscle. The extent of cross-innervation and absence of reinnervation was checked before fixation in the toxin.

## Cholinergic Receptors at Denervated Mammalian Motor End Plates

By

ERIK FRANK, <sup>1</sup>KAARE GAUTVIK and HENRIK SOMMERSCHILD

Received 3 March 1975

---

### Abstract

FRANK, E., K. GAUTVIK and H. SOMMERSCHILD *Cholinergic receptors at denervated mammalian motor end-plates* Acta physiol scand 1975 95 66-76

The number of acetylcholine receptors at normal and denervated end-plates in rat soleus muscles was studied using the binding of [<sup>125</sup>I] α-bungarotoxin as a quantitative assay. Normal end-plates bound several thousand times as much toxin as equal areas of extra-synaptic muscle membrane. After short-term denervation (up to 2.4 weeks) the extrafunctional binding increased, but there was no change in specific binding to the motor end-plate. Denervation for longer periods (up to 7 weeks) reduced binding sites at the end-plate by up to 60-70%. Direct electrical stimulation of these muscles for the entire period of denervation did not prevent the loss of functional binding sites even though it was adequate to abolish the increase in extrafunctional toxin binding. In contrast, denervated end-plates on muscle fibres cross-innervated by a foreign nerve at a distant location continued to bind normal amounts of toxin for over four months.

---

Embryonic mammalian skeletal muscle fibres are sensitive to acetylcholine (ACh) along their entire length. Shortly after birth, however, this sensitivity becomes restricted to the small area of synaptic contact between nerve and muscle (Diamond and Miledi 1962). When impulse activity in the motor nerve is blocked or the nerve itself is destroyed, ACh sensitivity again appears outside the synaptic region (Axelsson and Thesleff 1959; Lomo and Rosenthal 1972).

The development of high extra-synaptic sensitivity in the absence of a functional nerve supply might suggest that the nerve directly determines the distribution of ACh sensitivity. However, Lomo and Rosenthal (1972) have shown that direct electrical stimulation of a denervated muscle fibre also produces and maintains a low extra-junctional sensitivity. Under normal conditions the electrical and/or mechanical activity of the muscle can therefore be sufficient to account for the absence of sensitivity outside the synaptic region.

In the synaptic region itself, the formation and regulation of ACh receptors is only poorly understood. Unlike extra-junctional receptors, these receptors do not disappear during normal muscle activity. Initially a nerve can induce the appearance of junctional receptors and ACh

---

<sup>1</sup> Present address: Department of Pharmacology, Harvard Medical School, Boston, MA 02115, USA

## Results

### *trypticholine esterase staining of denervated end-plates*

Three types of denervated end-plates were adequately visualized with the acetylthiocholine stain, but the distribution and intensity of the reaction product were different than normal innervated end-plates. Representative examples are shown in Plate 1: an innervated end-plate is included for comparison. Denervated end-plates on muscle fibres that had been directly stimulated for up to 6 weeks from the time of denervation (Plate 1 B) are similar to those on fibres denervated for the same length of time but stimulated only for the final 1 1/2 weeks (Plate 1 C). The stain was as dense as at a normal end-plate (Plate 1 D) but the end-plates appeared to be much narrower. These esterase-stained end-plates were similar to those on denervated unstimulated fibres.

The staining of a denervated end-plate on a muscle fibre innervated elsewhere by another nerve is shown in Plate 1 A. Even after several months of denervation (and cross-innervation) an old end-plate stained well. Frequently there were long filamentous strands of reaction product extending from the end-plate region and often the intensity of the stain was less dense than normal.

### *Binding of labelled toxin*

The binding of [ $^{125}$ I]  $\alpha$ -bungarotoxin to end-plates and extrajunctional areas on a control and a denervated muscle is shown in Fig. 1. In normal muscles toxin binding was more than 10 times higher in the end-plate region than in the extra-junctional area. The actual end-plate occupies only a small fraction of the membrane in our "end-plate regions". If one assumes that toxin binding is normally confined to the synaptic area only (Hartzell and Fambrough 1972), the end-plate binds several thousand times more toxin than equal areas of non-endplate regions. The amount of toxin bound per end-plate was comparable to that found by other groups (Miledi and Potter 1971, Berg *et al.* 1972, Hartzell and Fambrough 1972). In order to investigate the effect of denervation alone, we measured toxin binding to two muscles denervated for a short time (1.4 and 2.4 weeks) that had not been stimulated at all. Fig. 1 illustrates the results from one of these muscles. Assuming that the extra-junctional binding is approximately uniform along the length of a denervated fibre we calculated end-plate specific toxin binding by subtracting out the binding to extra-junctional parts of the fibres in a manner similar to that used by Guth, Albers and Brown (1964) and Hall (1973) for cholinesterase. This assumption is qualitatively supported by autoradiographs prepared by Hartzell and Fambrough (1972, Fig. 1 B) and by binding data from Miledi and Potter (1971, Fig. 4) but apparently has not been rigorously tested. With this reservation, the binding to these end-plates (denervated 1.4 and 2.4 weeks) is not significantly different from the controls (see Fig. 4), in accord with earlier results (Hartzell and Fambrough 1972, Lomo and Rosenthal 1972).

To assess the long-term effects of denervation we stimulated the muscles for the final 1 1/2 weeks in order to abolish the high toxin binding to extrajunctional areas of the fibres. As shown in Fig. 2 the binding of [ $^{125}$ I]  $\alpha$ -bungarotoxin to end-plates on the denervated muscle fibres was reduced after a period of six weeks whether or not the muscles were chroni-



Fibres innervated by the foreign nerve were of normal diameter while those that were not cross-innervated were extremely atrophic. This provided an unambiguous identification of cross-innervated. Only end plates on normal sized fibres in regions of the muscle known to be cross-innervated were used for analysis. The absence of extra-junctional toxin binding provided further assurance that these were functionally innervated (see below). It was also important to verify that the foreign nerve had reinnervated the old soleus end plates. This was confirmed by looking for any nerve fibres in the soleus end-plate region, both in the whole muscle with a dissecting microscope and in teased fibre preparations examined with Nomarski optics. The esterase stain facilitated location of the end-plates in preparations. Two of six cross-innervated muscles appeared to have some foreign reinnervation end-plates and were not included in the results.

#### *Preparation of [ $^{125}$ I] $\alpha$ -bungarotoxin*

Purified  $\alpha$ -bu garotoxin (supplied by Miami Serpentarium) was assayed and migrated as a single band in 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS). The (20  $\mu$ g) was labelled with carrier-free  $\text{Na}^{125}\text{I}$  (3 mCi) using the chloramine T method (Green Hunter and Glover 1963). Bovine serum albumin was added after the iodination to a final concentration of 0.2%. After separation of the iodinated toxin from free  $^{125}\text{I}$  by gel filtration on Sephadex it was diluted in Krebs solution (final concentrations, mM:  $\text{Na}^+$  162,  $\text{K}^+$  5;  $\text{Ca}^{2+}$  2,  $\text{Mg}^{2+}$  1;  $\text{HCO}_3^-$  24,  $\text{Cl}^-$  148, glucose, 11) and stored in 15 one ml aliquots at  $-20^\circ\text{C}$ .

The addition of albumin to the toxin solutions complicated the measurement of specific activity. An estimate was made in the following way: Each aliquot of toxin had the same biological activity  $\mu\text{g}/\text{ml}$  solution of unlabelled toxin as determined by the rate of blockade of neuromuscular transmission in an isolated rat diaphragm muscle ( $\sim 45$  min). This suggests that at least 75% of the initial toxin recovered, and with this assumption the specific activity of 3 batches of labelled toxin varied between 4 and 14  $\cdot 10^4$  Ci/mole.

SDS polyacrylamide gel electrophoresis (10%) of the labelled toxin showed that more than 90% of the total radioactivity migrated as a single peak with a mobility corresponding to that of the unlabelled toxin. There was no detectable loss of binding activity after 10 weeks of storage.

#### *Binding of toxin to muscles*

Experimental and control soleus muscles were isolated under ether anaesthesia, dissected free of connective tissue, and incubated together in 1 ml of Krebs solution containing approximately 1  $\mu\text{g}$  of toxin for 2 h at room temperature. The solution was continually aerated with 5%  $\text{CO}_2$  and 95%  $\text{O}_2$ . Preliminary experiments indicated that binding of toxin to superficial end plates was complete in one hour. The muscles were washed overnight in a small volume of oxygenated Krebs solution at  $4^\circ\text{C}$  that was continuously stirred at 2 ml/min. After a short preincubation (10 min) in 1% glutaraldehyde in Krebs solution, the muscles were stained for ACh-esterase (Karnovsky 1964) and placed overnight in a 4% aldehyde fixative.

Small groups of superficial muscle fibres approximately 1 mm long and containing 1–15 fibres were teased out, and the number of stained end-plates were counted and compared with the number of fibres. The fibres were then dissolved in 0.5 ml of formic acid and the radioactivity was measured in a Packard gamma scintillation spectrometer (50 cpm background, 90% counting efficiency). In some experiments end-plates from the unoperated contralateral soleus muscle were always used to determine control binding levels, although the variations in amount of toxin binding to normal end-plates from different muscles were small. The results are presented as cpm per end-plate, corrected for isotopic decay of iodine and corrected for extra-junctional toxin binding (see below).

Similar lengths of fibres without end plates were used to measure non-end-plate binding. Binding to extra-junctional regions included the binding to extra-junctional ACh receptors and to other sites with some affinity for the toxin. It therefore represents an upper limit on background binding. In normal cross-innervated muscles, and in denervated muscles that had been adequately stimulated, extra-junctional binding was low enough (Fig. 2 and 3) so that it did not interfere with the measurement of binding to end-plates. Denervated-unstimulated muscles, however, always showed marked binding of toxin to the end-plate region (Fig. 1). Toxin binding to non-end-plate regions thus provided a useful control for the adequacy of electrical stimulation. Two stimulated muscles had a higher than normal non-end-plate binding and were not included in the results because they were probably not adequately stimulated. For all the others the amount of toxin bound per mm of fibre length was at least 10 times smaller than that associated with a single normal end-plate.



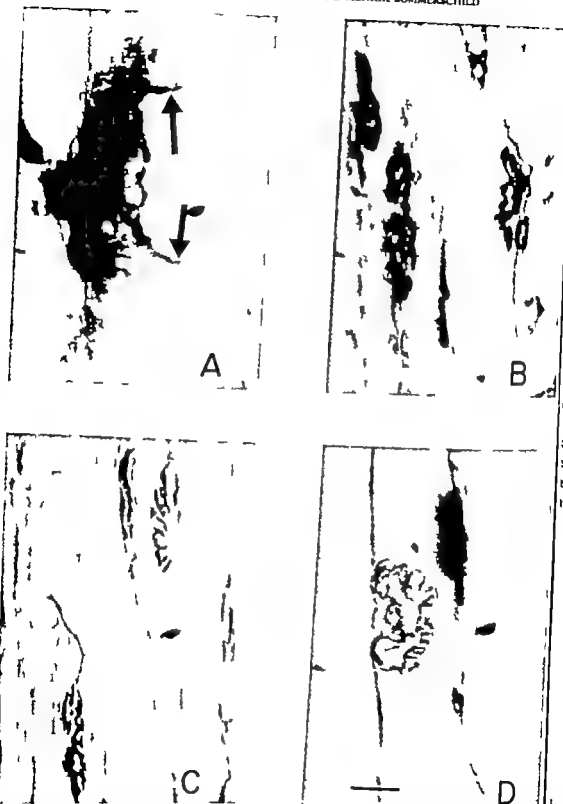


Plate 1 End-plates stained for ACh-esterase from soleus muscles that had been incubated with <sup>125</sup>I-acetylcholine. A. Denervated end-plate on muscle fiber that had been denervated and cross-innervated four months earlier. Arrow marks filamentous strands of reaction product extending from the end-plate. B. Denervated end-plates on fibers that had been denervated and chronically stimulated for six weeks. C. A normal innervated end-plate from an unoperated soleus muscle. The marker 20  $\mu$ m. D. A normal innervated end-plate from an unoperated soleus muscle. The marker 20  $\mu$ m.

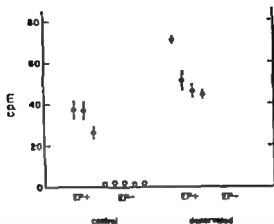


Fig. 1 Binding of  $^{125}\text{I}$ - $\alpha$ -bungarotoxin to normal soleus muscle (left) and soleus muscle denervated 10 days earlier (right). The filled circles in this figure and in Fig. 2 and 3 show the amount of bound toxin per end-plate on one mm length of muscle fibres (EP+). The open circles show the amount of bound toxin per 1 mm length of muscle fibre without end-plates (EP-). The increased binding to end-plate appears in the denervated muscle is probably explained by the high extra-junctional binding in this muscle. Error bars here and in Fig. 2 and 3 indicate  $\pm 2$  S.E. associated with the determination of radioactivity of each group of end-plates. The 5 points with the largest error bars are groups that contained 2 or 3 end-plates each. Control muscles in this and subsequent figures are the contralateral unoperated soleus muscles.

cally stimulated for the entire period of denervation. The results from two binding experiments in chronically denervated muscles are presented. In each case, the amounts of toxin (expressed as cpm/end-plate) bound to normal and denervated end-plates is shown as well as the background levels for extrajunctional portions of normal and denervated fibres. Error

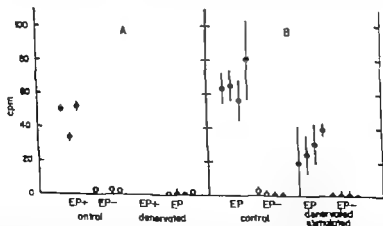


Fig. 2 Toxin binding to denervated end plates on two non-innervated muscles. Both experimental muscles had been denervated 6 weeks earlier. In A, the denervated muscle was electrically stimulated only for the final 12 days. In B, the muscle was stimulated continuously for the entire period. Neither muscle had significant number of extra-junctional binding sites, but in both muscles the denervated end-plates bound only half the normal amount of toxin. Filled circles in A represent 5-10 end-plates each, in B all groups except the one with the smallest error bar contained 1-3 end-plates.

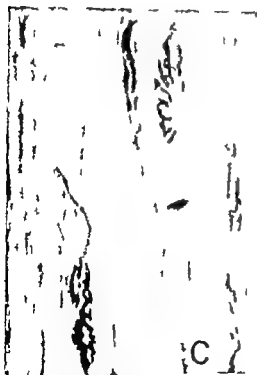


Plate 1 End-plates stained for ACh-esterase from soleus muscles that had been incubated in  $^{35}\text{S}$ -m-bungarotoxin. A. Denervated end-plate on a muscle fibre that had been denervated and cross-nervated 7 months earlier. Arrow marks filamentous strands of reaction product extending from the end-plate. B. Denervated end-plates on fibres that had been denervated and chronically stimulated for 6 weeks. C. As in B, but electrical stimulation was only for the final 2 days of the six week denervation period. D. A normal innervated end-plate from a nonoperated soleus muscle. The marker  $70\ \mu\text{m}$ , only at all pictures.

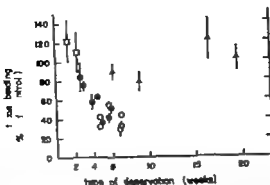


Fig. 4. Summary of results of toxin binding to denervated end-plates vs. time of denervation. Each point the average toxin binding to all measured end-plates on one muscle expressed as percentage of toxin binding to the normal end-plates on the contralateral muscle.  $\square$ , denervated, unstimulated muscles;  $\circ$ , denervated-stimulated muscles;  $\bullet$ , denervated muscles stimulated only for the final 1-2 weeks to reduce extrajunctional binding;  $\Delta$ , denervated muscles stimulated only for the final 1-2 weeks to reduce extrajunctional binding;  $\circ$ , cross-innervated muscles. Error bars show  $\pm 1$  S.E. of the mean, based on the variability of binding in different groups of end-plates from single muscles and taking into account the variable numbers of end-plates in each group. Denervation or denervation plus stimulation resulted in a slow loss of junctional binding sites over several weeks, whereas cross-innervation protected the sites at the old denervated end-plates for at least several months.

weeks for both groups. Denervated end-plates on cross-innervated fibres, on the other hand, retained their ability to bind toxin for at least several months.

### Atrophy

Muscle fibres that were innervated by the foreign nerve appeared normal at the end of the experimental period whereas denervated and denervated-stimulated fibres were somewhat atrophic. We tested for a possible correlation between atrophy and loss of junctional binding sites by weighing the dissected, aldehyde-fixed muscles. These measurements are shown in Fig. 5 percent of normal (paired) muscle weight is plotted against the time after denervation. Several denervated, unstimulated muscles are included for comparison.

The two muscles denervated for 1.4 and 2.4 weeks weighed only half as much as their paired controls, yet end-plates on these muscles bound approximately normal amounts of toxin (Fig. 4). After four weeks of denervation the weights of muscles stimulated only for the final 1 1/2 weeks or for the entire period had also fallen to about half. It was therefore not possible to assess the reduction in number of junctional binding sites independently of muscle atrophy at these later times.

### Discussion

#### Toxin binding to end-plates on denervated muscles

There was a considerable reduction in the number of toxin binding sites at end-plates on denervated muscle fibres with time. The electrical stimulation parameters employed in the present study apparently had little or no effect on the loss of toxin binding sites occurring at denervated end-plates. Muscles stimulated continuously from the time of denervation

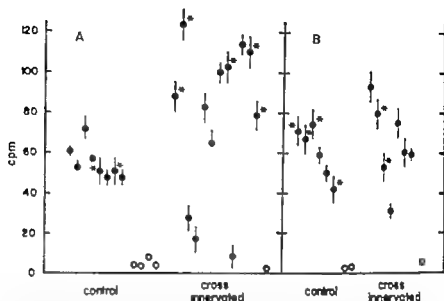


Fig. 3 Preservation of toxin binding to denervated end-plates on cross-innervated muscle fibres. The experimental muscle in A was denervated and cross-innervated (see Methods) 16.6 weeks earlier in B 19.6 weeks earlier. There was a greater than normal variation in the binding to individual end-plates, but on the average denervated end-plates in these muscles bound just as much toxin as normal innervated end-plates did. Each point marked  $\bullet$  represents a single end-plate other groups contained 2-7 end-plates.

bars indicate the uncertainty in the measurement of radioactivity of each group of end-plates. The experimental muscle shown in Fig. 2 A was denervated for a total of 5.7 weeks and was stimulated for the last 12 days in order to eliminate extrajunctional binding, while in Fig. 2 B the muscle was stimulated continually from the time of denervation for a total of 6 weeks. Chronic stimulation provided no protection against the reduction in toxin binding in both cases the end-plate binding fell to about half of the control values.

The loss of binding sites from end-plates denervated for several weeks was in sharp contrast to results obtained for denervated end-plates on fibres innervated by another nerve but at a distant location. Two examples are presented in Fig. 3. The end-plates on one muscle, Fig. 3 A, had been denervated for 16.1 weeks and in Fig. 3 B for 19.6 weeks. On the average the original ("old") end-plates in these muscles bound just as much toxin as normal end-plates did, despite the fact they had been denervated for over four months. Denervated end-plates on fibres cross-innervated for shorter times also bound normal amounts of toxin (see Fig. 4). When single end-plates were dissected out and assayed, there was considerable variation in the amount of toxin bound to each end-plate (points marked  $\bullet$  in Fig. 3). Some bound more toxin than normal while others bound almost none, even though all the fibres were cross-innervated (see Methods). There is a similar variability in the ACh sensitivity of denervated end-plates on cross-innervated muscle fibres (Frank *et al.* 1975).

The combined results of toxin binding to all denervated end-plates are summarized in Fig. 4. Binding is expressed as a percentage of the average binding to normal soleus end-plates of the same rat. There is a slow reduction in the number of toxin binding sites on denervated end-plates both for fibres stimulated from the time of denervation and fibres stimulated only at the end of the denervation period. On the average toxin binding fell 60-70% in four

is also of interest that toxin binding was greatly reduced at denervated end-plates on one of the cross-innervated muscle fibres. This variability between individual end-plates may be related to the time at which cross-innervation of a particular fibre actually occurs. For example, when a foreign nerve axon innervates a denervated muscle fibre as a new location it might preserve the current level of esterase activity and number of toxin binding sites at an old end-plate. Alternatively it may be that over long periods of time innervation at a new site is simply insufficient to prevent changes in the original end-plate and that different fibres undergo these changes at different rates.

#### *Stable mechanisms of junctional receptor maintenance*

There was a striking difference between the amount of toxin bound to denervated end-plates in muscles with and without cross-innervation. One factor that might explain this difference is that denervated muscles underwent atrophy whether they were electrically stimulated or not whereas cross-innervated fibres remained normal in size. Loss of toxin binding sites might occur *pari passu* with a general reduction in muscle size.

The two muscles denervated for only 1.4 and 2.4 weeks indicate that there is not a constant relationship between junctional receptors and muscle weight. Although the muscles had lost 1/3 of their original weight the end-plates bound apparently normal amounts of toxin. These data, however, are still compatible with the idea of a delayed loss of junctional receptors following general muscle atrophy. The experiments with denervated-stimulated muscles did not resolve this issue because electrical stimulation did not completely block muscle atrophy. The stimulation parameters were sufficient to suppress extra-junctional toxin binding, but it is possible that more adequate stimulation might also preserve junctional binding sites.

An alternative possibility is that the foreign nerve exerted some direct trophic effect on the original, denervated end-plates independent of its role in activating the muscle. Smith *et al.* (1966) suggested a similar hypothesis to explain the preservation of ACh esterase activity at denervated end-plates on cross-innervated muscles. Whatever the mechanism turns out to be, the persistence of junctional receptors at denervated end-plates on cross-innervated muscle fibres is remarkable.

We pleasure to acknowledge the advice provided by T. Lomo, Drs J. K. S. Jansen and D. E. Van der Meulen during all phases of this work. E. F. was supported by a postdoctoral fellowship from the Muscular Atrophy Association of America, Inc.

#### References

- ALLISON, J. and S. TREKLEY. A study of supersensitivity in denervated mammalian skeletal muscles. *J. Physiol. (Lond.)* 1959, 147, 178-193.
- IRBY, O. H., R. B. KELL, P. B. BABOINET, P. WILLIAMSON and Z. W. HALL. Binding of  $\alpha$ -bungarotoxin to acetylcholine receptors in mammalian muscle. *Proc. nat. Acad. Sci. (Wash.)* 1972, 69, 147-151.
- JAMOND, I. and R. MILNER. A study of foetal and newborn rat muscle fibres. *J. Physiol. (Lond.)* 1962, 112, 393-408.
- JANSEN, J. K. S. and J. JANSEN. T. LOMO and R. WESTGAARD. The innervation between foreign and original motor nerves innervating the soleus muscle of rats. *J. Physiol. (Lond.)* 1973, in press.
- JENNINGS, F. C., W. M. HUNTER and J. S. GLOVER. The preparation of  $^{125}$ I-labelled bovine growth hormone of high specific radioactivity. *Biochem. J.* 1963, 89, 114-123.



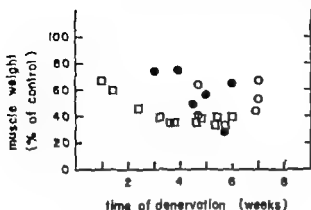


Fig. 5. Atrophy of denervated and denervated stimulated muscles vs. time. □ denervated not stimulated (some of this data was provided by T. Lomo, personal communication). ● denervated muscles stimulated only in the last 1½ weeks. ● denervated-stimulated muscles. Atrophy is expressed as the weight of aldehyde-fixed denervated muscles compared to the contra lateral unoperated muscles. Stimulation provided some protection against atrophy but the protection was incomplete.

had to be compared to muscles stimulated for the final 1½ weeks nevertheless a pronounced preserving effect of continuous stimulation on the ACh receptors would probably have resulted in some discrepancy between the binding to the two types of end-plates. No such discrepancies were observed. A simple hypothesis that is consistent with the data is that toxin binding to denervated end plates falls steadily over several weeks whether the denervated muscle is stimulated or not.

ACh esterase associated with denervated end-plates also declines with time. Guth *et al.* (1964) reported a fall to 25% in two weeks for the rat's sternomastoid muscle and Hall (1973) found that seven days after denervation end-plate specific esterase had fallen to 21% of normal in the diaphragm. The early loss of esterase was somewhat faster than the decline of muscle weight (Guth *et al.* 1964). Thus after denervation the loss of end-plate esterase begins before fibre atrophy and subsequent loss of toxin binding sites. After four to six weeks, however levels of esterase activity and numbers of binding sites are reduced to approximately the same extent (Guth *et al.* 1964, Guth, Zalewski and Brown 1966).

#### *Toxin binding to cross-innervated muscles*

In contrast to the loss of toxin binding sites from end-plates on denervated muscles, no end plates on cross-innervated muscle fibres showed no diminution of toxin binding even after four months. The distribution of these binding sites is not known microscopically. At normal end-plates, Hartrell and Fambrough (1972) have shown that toxin binding is highly restricted to the synapse itself as revealed by combined autoradiography and ACh esterase stains. In view of the faint filamentous appearance of the esterase stain at some of the denervated end-plates on cross-innervated fibres, it would be interesting to know the microscopic distribution of toxin binding sites and to compare at single end plates the number of toxin sites with quantitative determinations of esterase activity.

The maintenance of normal toxin binding characteristics of the denervated end-plate on cross-innervated muscles is reminiscent of the maintenance of old end-plate esterase activity on cross-innervated sternomastoid muscles of rats reported by Guth *et al.* (1966). These authors found that after 8 weeks of denervation and cross innervation the esterase activity in the old end-plate region was half that of normal controls, whereas with denervation

## Differential Effects of Temperature on Activity of LDH from Seal and Sheep Skin

By

ARNOLDUS SCHYTTE BLIK, EDVARD B. MØRRELT and HANS J. GRAY

Received 4 March 1975

### Abstract

X, A. S., E. B. MØRRELT and H. J. GRAY. *Differential effects of temperature on activity of LDH from seal and sheep skin.* Acta physiol. scand. 1975. 95. 77-82.

1 homeothermic seal possesses heterothermic skin, while the skin of the sheep behaves as truly homeothermic tissue. A new method for skin homogenization is described. The effect of temperature on catalytic behaviour of seal and sheep skin LDH has been investigated by fluorimetric activity measurements, with results presented as Arrhenius plots. The seal skin enzyme had ten times higher activity at 1 temperatures as compared to the sheep skin. The mechanisms responsible for this different behaviour 1 discussed

1 cold 1 ster the skin temperature of arctic seals approaches that of the aqueous environment (Irving and Hart 1957). While basking in the sun, the skin of such animals frequently 1 pores temperatures exceeding 40°C (Grimsland 1970). This means that the homeothermic 1 al possesses a heterothermic skin. Conversely sheep skin is protected by a thick woolly 1 out, and behaves like truly homeothermic tissue (cf. Fig. 2).

In order to retain metabolic function in a wide heterothermic range, the seal skin enzymes should possess catalytic activities quantitatively different from those of the homeothermic sheep skin. As a possible candidate for such adaptive change we therefore chose to investigate possible differences in temperature sensitivity of lactate dehydrogenase (E.C. 1.1.1.27) from the skin of these animals. This key glycolytic enzyme was selected because 1 is crucial to skin survival, both under anaerobic (seal during diving) as well as aerobic conditions

### Methods

Subcutaneous and rectal temperatures of an adult female hooded seal (*Cystophora cristata*) were recorded by use of thermocouples connected to a Spedonox multi-channel recorder. The studies were carried out aboard a sailing vessel in the North Atlantic Ocean, off Jan Mayen. Ambient temperature was -5°C and the wind speed 13 m/s. The recordings were continued with the animal submerged (diving) in sea water of 1.5°C.

- GUTH, L., R. W. ALBERS and W. C. BROWN, Quantitative changes in cholinesterase activity of denervated muscle fibers and sole plates. *Exp. Neurol.* 1964. 10. 236-250.
- GUTH, L., A. A. ZALEWSKI and W. C. BROWN, Quantitative changes in cholinesterase activity of denervated sole plates following implantation of nerve into muscle. *Exp. Neurol.* 1966. 16. 136-147.
- HALL, Z. W. Multiple forms of acetylcholinesterase and their distribution in endplate and nonendplate regions of rat diaphragm muscle. *J. Neurobiol.* 1973. 4. 343-361.
- HARTZELL, H. C. and D. M. FAMBROUGH, Acetylcholine receptors: distribution and extrajunctional density in rat diaphragm after denervation correlated with acetylcholine sensitivity. *J. gen. Physiol.* 1971. 58. 248-262.
- HOFFMAN, H. A study of the factors influencing innervation of muscles by implanted nerves. *Amer. J. exp. Biol. med. Sci.* 1951. 29. 289-308.
- KARNOVSKY, M. J. The localization of cholinesterase activity in the rat cardiac muscle by electron microscopy. *J. Cell Biol.* 1964. 23. 217-232.
- LINNO, T. and J. ROSENTHAL, Control of ACh sensitivity by muscle activity in the rat. *J. Physiol. (Lond.)* 1972. 221. 493-513.
- MILEDI, R. and L. T. POTTER, Acetylcholine receptors in muscle fibres. *Nature (Lond.)* 1971. 233. 99-102.

## Differential Effects of Temperature on Activity of LDH from Seal and Sheep Skin

By

ARNOLDUS SCHYTTE BLØD, EDVARD B. MØRSELY and HANS J. GRAY

Received 4 March 1975

### Abstract

BLØD, A. S., E. B. MØRSELY and H. J. GRAY. *Differential effects of temperature on activity of LDH from seal and sheep skin*. Acta physiol. scand. 1975. 95. 77-82.

A homeothermic seal possesses a heterothermic skin, while the skin of the sheep behaves as a truly homeothermic tissue. A new method for skin homogenization is described. The effect of temperature on catalytic behaviour of seal and sheep skin LDH has been investigated by fluorimetric activity measurements, with results presented as Arrhenius plots. The seal skin enzyme had ten times higher activity at 5 temperatures as compared to the sheep skin. The mechanisms responsible for this different behaviour is discussed.

In cold water the skin temperature of arctic seals approaches that of the aqueous environment (Irving and Hart 1957). While basking in the sun, the skin of such animals frequently attains temperatures exceeding 40°C (Brittland 1970). This means that the homeothermic seal possesses a heterothermic skin. Conversely sheep skin is protected by a thick woolly coat, and behaves like a truly homeothermic tissue (cf. Fig. 2).

In order to retain metabolic function in a wide heterothermic range, the seal skin enzymes should possess catalytic activities quantitatively different from those of the homeothermic sheep skin. As a possible candidate for such adaptive change we therefore chose to investigate possible differences in temperature sensitivity of lactate dehydrogenase (E.C. 1.1.1.27) from the skin of these animals. This key glycolytic enzyme was selected because it is crucial to skin survival, both under anaerobic (seal during diving) as well as aerobic conditions.

### Methods

Subcutaneous and rectal temperatures of an adult female hooded seal (*Cystophora cristata*) were recorded by two thermocouples connected to a Speedomax multi-channel recorder. The studies were carried out aboard a fishing vessel in the North Atlantic Ocean, off Jan Mayen. Ambient temperature was -5°C and the wind speed 13 m/s. The recordings were continued with the animal submerged (diving) in sea water at 15°C.

Recordings of subcutaneous temperatures were also gathered from domestic sheep exposed to temperatures ranging from  $-20$  to  $+40^{\circ}\text{C}$  in a temperature controlled room.

#### *Animals and samples*

Ringed seals (*Phoca hispida*) and domestic sheep were used in this study. The seals were obtained from Bay Spitzbergen, and the sheep from a farm outside Oslo, Norway. Skin samples were excised from the hind flipper (leg) of the seals and from the middle back of the sheep, and frozen in liquid nitrogen for 10 min after sacrificing the animal. The samples were thereafter kept at  $-85^{\circ}\text{C}$ .

#### *Homogenization*

The samples were cut in sections  $10\mu\text{m}$  thick on a Jungo microtome mounted in a Linds cryostat. About 100 sections were assembled in a high speed centrifuge tube containing 1 ml  $0.1\text{ M Na}_2\text{HPO}_4$  buffer pH 7.4. The tubes were incubated at room temperature for 10 min and centrifuged for 1 h at  $50\,000 \times g$  in a Sorvall RC2 B centrifuge. The supernatants were used as enzyme source immediately after centrifugation.

#### *Protein*

Protein was determined according to Lowry *et al.* (1951). Electrophoresis was performed according to Dietz and Lubrano (1967), as modified by Dietz *et al.* (1970) and by Fritz *et al.* (1970).

#### *Enzyme assay*

Maximal LDH activity was determined by spectrophotometric assessment of rates of oxidation of NADH (Sigma) at different substrate (pyruvate, Sigma) concentrations. The measuring wavelength was 340 nm. The incubation mixture contained  $0.1\text{ M}$  sodium phosphate buffer pH 7.4, pyruvate in concentrations ranging from  $30\mu\text{M}$  to  $10\text{ mM}$ ,  $4\text{ mM}$  NADH, and  $0.1\text{ ml}$  of the supernatant, in a total volume of  $1\text{ ml}$ .

#### *Effect of temperature on LDH catalytic behaviour*

Arrhenius plots for seal- and sheep skin LDH were constructed from the specific activity values at different temperatures. For this purpose LDH activity was measured fluorimetrically using a mixture containing the following final concentrations of components:  $5\mu\text{M}$  NADH,  $100\text{ mM}$  tris-amine-HCl buffer pH 7.2, and enzyme in  $0.1\text{ M}$  sodium phosphate buffer. The pyruvate concentration was so adjusted that enzyme activity remained within 10% of maximal activity under all experimental conditions. Oxidation of NADH was followed with an Eppendorf photometer (Model III 1911) with a photometer attachment and recorder adapter for signal amplification. The reaction was started by the addition of pyruvate and internally standardized by adding  $0.1\text{ nmol}$  NADH prior to termination of the reaction. The chamber holding the cuvette was thermocontrolled by the electronic device described by Nishikawa *et al.* (1974).

## Results

While resting in air at  $-5^{\circ}\text{C}$ , the hooded seal revealed subcutaneous temperatures of  $13.2 \pm 0.2^{\circ}\text{C}$  and  $24.2 \pm 1.0^{\circ}\text{C}$  respectively at two different locations (the first exposed to a wind speed of  $13\text{ m/s}$ , the second leeward). Rectal temperature was  $36^{\circ}\text{C}$ . Upon diving, the animal displayed a drop in subcutaneous temperature. Fig. 1 illustrates the changes in subcutaneous temperature during a dive in ice water after a series of submersions.

Regardless of ambient temperature, the subcutaneous temperature of the sheep was constant at  $38^{\circ}\text{C}$  (Fig. 2). This value is close to the animal's deep body temperature.

The electrophoretic analysis of the isoenzyme pattern exposed twice as much M<sub>1</sub> in the seal skin enzyme as in the sheep skin (Table I). The seal skin enzyme, furthermore exhibited maximum activity at the higher substrate concentration (Table I) as would be expected from the isoenzyme patterns (Kaplan *et al.* 1968).

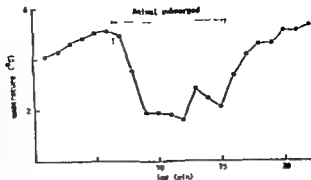


Fig. 1. Subcutaneous temperature of hooded seal in response to changes in ice water after exposure to ambient temperatures of  $5^{\circ}\text{C}$ , following series of 11 submersions. Animal cold dead (arctic winter).

Fig. 3 illustrates the effect of temperature on total LDH catalytic activity within the range  $-42.9^{\circ}\text{C}$ . The Arrhenius plots reveal that specific enzyme activity is higher in the seal skin enzyme throughout this range. At low temperature ( $3.5^{\circ}\text{C}$ ), the sheep skin enzyme is 10 per cent less active on a protein basis. This difference in catalytic behaviour becomes strikingly apparent from the calculated activation energies, that of the sheep skin requiring higher energy input. Moreover the Arrhenius plot of the seal skin enzyme is characterized by an abrupt decrease in activation energy as the temperature is lowered beyond  $20^{\circ}\text{C}$ . No such variation from the behaviour predicted by the Arrhenius equation is observed with sheep skin LDH.

### Discussion

Several studies (e.g. Irving and Hart 1957 and Ray and Fay 1967) have indicated that the seal skin has to tolerate temperatures below  $20^{\circ}\text{C}$  for prolonged periods in the course of arctic winter. Moreover during diving the blood flow through the vascular bed of the skin is arrested, and energy production consequently has to proceed anaerobically at temperatures close to  $0^{\circ}\text{C}$  (Fig. 1). The sheep skin, on the other hand, seems to be well circulated

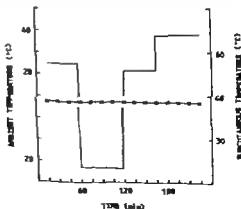


Fig. 2. Subcutaneous temperature of cold adapted sheep (■), in response to changing ambient temperature (—).

TABLE I Percentage distribution of LDH isoenzymes obtained electrophoretically from homogenized seal and sheep skin. The table also includes the calculated M-subunit content and the seal (pyruvate) concentration of maximal catalytic activity of the enzyme.

	Percentage distribution of isoenzymes					Percent M subunit	Substrate concentration at maximal activity (mmol/l)
	Isoenzyme number						
	1	2	3	4	5		
Seal	—	6	22	28	44	78	$10^{-4}$
Sheep	26	16	43	11	4	38	$3 \cdot 10^{-4}$

and maintained at a temperature close to that of the central core, independent of the environment (Fig. 2)

This functional difference between the two types of skin tissue is reflected in the differently different catalytic behaviour of their lactate dehydrogenases, an important enzyme in the intermediary metabolism. Thus, the enzyme from seal skin exhibited an activity times greater than that of sheep skin at low temperatures (Fig. 3)

As to the mechanisms which allow this different behaviour it is apparent from the Arrhenius plots that the sheep skin enzyme calls for much higher activation energies than the seal skin enzyme throughout the biologically important temperature range (Fig. 3). The break in the plot makes this difference particularly acute at low temperatures where the seal skin enzyme is characterized by an activation energy only half that of sheep skin enzyme.

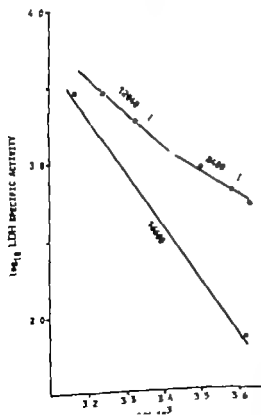


Fig. 3 Arrhenius plots for seal (●) and sheep skin LDH showing the differential effect of temperature on the catalytic behaviour of the two LDH specific activities. The figures on each plot represent activation energy (cal) calculated over a given temperature range. The break in the plot for seal skin LDH occurs at a range of temperatures 3.5–4.2°C.

ch breaks in the Arrhenius plot have occasionally been shown to be a result of direct effect of temperature on protein structure (e.g. Massey 1953). Irrespective of explanation, plots of this peculiar shape reflect catalytic properties of great benefit for enzymes and to function for prolonged periods at very low temperatures.

According to Fritz (1967), the LDH 5 ( $M_2$ ) from rabbit muscle increased its activity more than 200% as the pH of the medium decreased from 7.8 to 6.4. The LDH from seal consists almost exclusively of  $M$ -subunits (Table I). It therefore seems likely that a large increase in enzyme activity can take place in the seal skin in response to the low pH reached during diving. Massey (1953) has demonstrated that a similar effect on the enzyme rate is caused by changes in activation energy.

The higher enzyme activity in the seal skin at low temperatures, as compared to the sheep, may also result from an enhanced enzyme-substrate affinity as reported for the heteromeric (liver) LDH of the common seal (Somero and Johansen 1970).

Superficially the observed isoenzyme pattern of the seal skin seems to agree with postulated catalytic function under anaerobic conditions (Everse and Kaplan 1973). However, demonstrated by Berg and Blix (1973), different cell types of the same tissue may exhibit different isoenzyme patterns. The skin is a tissue of extreme cellular heterogeneity and available data is insufficient to allow discrimination of differing metabolic activities in the various cell types. It follows that it is not yet possible to decide whether the observed differential effects of temperature on LDH activity (Fig. 3) is a direct consequence of the different isoenzyme patterns of the two types of skin.

Whatever the mechanism, the skin is maintained in a functional state throughout the wide temperature range ( $-4$  to  $40^\circ\text{C}$ ) by the seal although, as demonstrated by Feltz and Fay (1966), epidermal cells from great many species of Pinnipeds require temperatures above  $10^\circ\text{C}$  for proliferation. The survival of the seal skin during arctic winter therefore, has to depend on its unusual ability to endure prolonged (2-6 months) hypothermia of  $4^\circ\text{C}$  (Feltz and Fay 1966).

We are grateful to Prof. O. Lundberg and Dr J. Valian of the Wenner-Gren Institute, Karol. Universitet Stockholm for generously providing the facilities and equipment necessary for fluorimetric measurements. This investigation was supported by the Norwegian Council for Science and the Finnmark, the Norwegian Svalbard Council and Norsk Polarisering (Norwegian Polar Institute).

## References

- BLIX, J. and A. S. BLIX. Distribution of lactate dehydrogenase isoenzymes in rat liver cells. *Nature New Biol. (Lond.)* 1973. 245: 239-240.
- FELTZ, A. A. and T. LUNDAHL. Separation and quantitation of lactate dehydrogenase isoenzymes by disc electrophoresis. *Analyt. Biochem.* 1967. 20: 246-257.
- FELTZ, A. A., T. LUNDAHL and H. M. RUMSTEDT. Disc electrophoresis of lactate dehydrogenase isoenzymes. *Chim. clin. Acta* 1970. 27: 223-232.
- EVERSE, J. and K. O. KAPLAN. Lactate dehydrogenase: Structure and function. *Advan. Enzymol.* 1973. 37: 61-113.
- FELTZ, E. T. and P. H. F. Thermal requirements *in vitro* of epidermal cells from seals. *Cryobiology* 1966. 3: 261-264.
- FELTZ, P. J. Rabbit lactate dehydrogenase isoenzyme: Effect of pH on activity. *Science* 1967. 156: 82-83.
- FELTZ, P. J., W. J. MORTIMER, E. L. WHITE and E. S. VERNILL. Comparative study of methods for quantitation of lactate dehydrogenase isoenzymes. *Analyt. Biochem.* 1970. 36: 443-453.



- IRVING, L. and J. S. HART. The metabolism and insulation of seals as bare-skinned mammals in cold air. *Canad. J. Zool.* 1957, 35: 497-511.
- KAPLAN, N. O., J. EVERSE and J. ADMIRAAL. Significance of substrate inhibition of dehydrogenases. *N. Y. Acad. Sci.* 1968, 151: 400-412.
- LOWRY, O. H., H. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951, 193: 265-275.
- MARLEY, V. Studies on fumarate: The effect of temperature. *Biochem. J.* 1953, 53: 72-79.
- NÄSLUND, P. B., LILJEVÄN and L. ABRAHAMSSON. High resolution analyzer of thermal density. *Analyt. Biochem.* 1974, 57: 211-218.
- GRITLAND, N. A., Energetic significance of absorption of solar radiation in polar homeotherms. *Antarctic Ecology* (Ed. M. W. Holdgate), 1: 464-470. Acad. Press, 1970.
- RAY, C. and F. H. FAY. Influence of climate on the distribution of walruses. II. Evidence from physiological characteristics. *Zoologica* 1968, 53: 19-32.
- SOMERO, G. N. and K. JOHANSEN. Temperature effects on enzymes from homeothermic and heterothermic tissues of the harbor seal. *Comp. Biochem. Physiol.* 1970, 54: 131-136.

## Effect of Spinal Sympathetic Blockade upon Local Regulation of Blood Flow in Subcutaneous Tissue

By

O. HENRIKSEN and T. ALSTER

Received 5 March 1975

---

### Abstract

HENRIKSEN, O. and T. ALSTER. *Effect of spinal sympathetic blockade upon local regulation of blood flow in subcutaneous tissue* Acta physiol. scand. 1975 95 83-88

The influence of spinal sympathetic blockade upon local regulation of blood flow in subcutaneous adipose tissue was investigated in six subjects. The effect of changes in orthostatic pressure on blood flow in subcutaneous tissue in the arm and distal forearm was measured before and after sympathetic blockade obtained by epidural anaesthesia in 4 subjects and by bilateral sympathectomy in 2 patients suffering from axonal hyperhidrosis. Blood flow in subcutaneous tissue measured by  $^{86}\text{Kr}$  washout technique decreased about 40 per cent when the limb was lowered, and remained constant during 30 cm elevation. This was true both before and after the blockade, though in one of the patients, the orthostatic decrease in blood flow was less pronounced 24 h after sympathectomy. Hence central sympathetic reflexes do not alter local flow-rate changes of blood flow in subcutaneous tissue. These changes therefore are most likely due to local mechanisms.

---

Blood flow in subcutaneous adipose tissue of a limb remains almost constant when the region studied is displaced within approximately 20 cm above and 20 cm below heart level. Outside these levels, blood flow decreases (Henriksen *et al.* 1973, Pærskov and Henriksen 1973). During elevation, perfusion pressure decreases as venous pressure remains constant. This indicates that blood flow is kept constant by a decrease in vascular resistance. When the region studied is positioned more than 20 cm below heart level, a decrease in blood flow must be due to an increase in vascular resistance as arterial and venous pressure increases in parallel below heart level.

These changes in vascular resistance could be due to changes in sympathetic vasoconstrictor activity. Stimulation of sympathetic nerves induces vasoconstriction in subcutaneous adipose tissue of the dog (Öberg and Rosell 1967). The contribution of vessels in the skin to changes in resistance induced by postural changes is still a matter of debate. Roddie and Sheperd (1956, 1957), Roddie *et al.* (1958), concluded that skin vessels do not contribute to the regulation of the total resistance during orthostatic pressure changes and changes in arterial baroreceptor activity (Roddie and Sheperd 1956, Roddie and Sheperd 1957, Roddie

*et al* 1958). This conclusion has been supported by others (Dellus *et al* 1972, Hanley and Skinner 1971, Ngal *et al* 1969). However, other investigators found that skin does contribute (Mosley 1969, Beiser *et al* 1970, Rowell *et al* 1973).

The present study deals with the possible role of sympathetic nerves in the changes in vascular resistance during local orthostatic pressure changes (Henriksen 1973, Paaske and Henriksen 1973).

### Experimental procedure

**Epidural anesthesia.** The experiments were carried out on 4 subjects referred to hospital for hernia to be performed in epidural anesthesia. The subjects were placed in the examination room 90 min before the measurements were started. Room temperature was about 21°C and remained constant throughout the investigation. Skin temperature was measured. The subjects were placed in a supine position, but supported at the crux and heel so that the areas under study could be placed at levels below or at the mid-axillary line.

<sup>125</sup>Xenon was applied to subcutaneous tissue by means of the intracutaneous injection technique (Sjörsten 1971). About 90 min after the injection <sup>125</sup>Xe is exclusively located in subcutaneous tissue. This isotope was chosen in order to avoid trauma to the subcutaneous tissue. The areas under study were located at the fibular head and at the lateral malleolus. Both legs were investigated.

A catheter was placed in the epidural space corresponding to the third lumbar vertebra. Blood flow in subcutaneous tissue was measured consecutively with the legs placed horizontally in such a way that the proximal and distal depots were lowered 30 and 40 cm below the mid-axillary line respectively and then returned to the horizontal position. Hereafter the legs were elevated so that the depots were raised 30 cm above the mid-axillary line. Finally the blood flow was measured with the legs placed horizontally. Each measurement lasted about 8 min.

Marcaïn 0.5%, without adrenaline, was injected into the epidural space through the catheter. Volume was 1.5 ml per segment. The blockade was extended to involve the 6th to 8th thoracic segments. Increase in skin temperature measured on the big toe (Ros *et al* 1973), disappearance of the sympathetic galvane reflex (Daos *et al* 1963), and abolished sweat production, tested by the anhydria test (Lundberg *et al* 1960), were taken as evidence of an effective sympathetic blockade. After termination of the investigation the sweat test was negative. In order to avoid a decrease in arterial blood pressure, isotonic saline was infused before the blockade was started. Arterial blood pressure was monitored and remained constant throughout the investigation.

After the sympathetic blockade had been achieved, measurements of blood flow in the same area were repeated as described above. The plasma concentration of adrenaline and nor-adrenaline was measured before and after the sympathetic blockade. Samples of 20 ml blood were taken from a superficial femoral artery for determination according to Valeri *et al* (1973), using an Amico-Bowman fluorescence fluorometer (Type D 223-62153).

### Bilateral sympathectomy

Two patients suffering from manual hyperhidrosis were examined before and 24 h after partial bilateral sympathectomy was performed. Blood flow in subcutaneous tissue in the distal part of the forearm was measured with the investigated area placed at reference level (jugular notch) and lowered 30 and 40 cm below the jugular notch.

A section of the sympathetic chain with one ganglion above and one below the third rib was removed. After the operation the sweat test was negative.

### Calculations

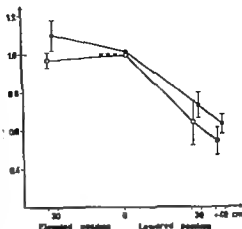
Blood flow was measured by means of the <sup>125</sup>Xe washout technique (Sjörsten 1971).

The perfusion coefficient,  $f$ , was calculated from the Kety equation

$$f = k \lambda / 100 (\text{ml}/100 \text{ g min})$$

where  $k$  denotes the rate constant of the washout function ( $\text{min}^{-1}$ ) and  $\lambda$  the adipose tissue to blood partition coefficient ( $\text{ml/g}$ ) (Kety 1951). In the calculations, a  $\lambda$ -value of 10 was used (Sjörsten 1971). The

Average (Flow/flow)



1. Relative change in mean blood flow ( $\text{avg } f_{\text{rel}}$ ) in subcutaneous tissue during post- changes of  $f_{\text{rel}}$  before and after sympathetic blockade. O—before, ●—after. The  $n$  denotes number of experiments. The vertical lines denote one standard error of the  $\text{avg}$ .

$t$  site constant,  $k$ , was computed from the regression line of the logarithmically transformed count  $n$  (corrected for background activity) by means of the method of least squares.

#### Statistics

Mean perfusion coefficient obtained at reference level ( $f_{\text{ref}}$ ) was compared to mean perfusion coefficient obtained at test level ( $f_{\text{test}}$ ) by means of Student's  $t$ -test for paired samples.  $f_{\text{test}}/f_{\text{ref}}$  and the concentration in plasma of adrenaline and nor-adrenaline, measured before and after sympathetic blockade, are compared by the randomization test for paired samples, respectively  $f_{\text{ref}}$  measured before and after sympathetic blockade was compared by the randomization test for independent samples. As level of significance was chosen 0.05.

### Results

**Spinal anesthesia.** The results are shown in Fig. 1. In all cases an increase in skin temperature of about 3–5°C was found after epidural anesthesia. Blood flow measured with the leg placed horizontally before and after sympathetic blockade is shown graphically in Fig. 2. Mean  $f_{\text{rel}}$  increased significantly from 6.0 ml/100 g min (range 1.3–10.2) to 8.4 ml/100 g min (range 2.9–16.9) after the blockade ( $p < 0.005$ ). The average concentration in plasma of adrenaline increased from 53 ng/l plasma (range 0–257) before to 256 ng/l plasma (range 0–420) after sympathetic blockade. Normal range: 0–337 ng/l plasma,  $p < 0.05$ .

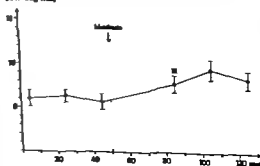
Average  $f_{\text{rel}}$   
(ml/100g min)

Fig. 2. Average blood flow in subcutaneous tissue measured with the leg placed horizontally before and after epidural anesthesia. The figures denote the number of experiments. The vertical lines denote one standard error of the mean.

*et al.* 1958). This conclusion has been supported by others (Delius *et al.* 1972, Hankey Sach and Skinner 1971, Ngai *et al.* 1969). However, other investigators found that skin vessels do contribute (Mosley 1969, Beaser *et al.* 1970, Rowell *et al.* 1973).

The present study deals with the possible role of sympathetic nerves in the observed changes in vascular resistance during local orthostatic pressure changes (Henriksen *et al.* 1973, Paaske and Henriksen 1973).

### Experimental procedure

**Epidural anesthesia.** The experiments were carried out on 4 subjects referred to hospital for operations to be performed in epidural anesthesia. The subjects were placed in the examination room 90 min before the measurements were started. Room temperature was about 21°C and remained constant during the investigation. Skin temperature was measured. The subjects were placed in a supine position with the legs supported at the knees and heel so that the areas under study could be placed at levels below or above the mid-axillary line.

<sup>133</sup>Xenon was applied to subcutaneous tissue by means of the intracutaneous injection technique (Sejrsen 1971). About 90 min after the injection <sup>133</sup>Xe is exclusively located in subcutaneous tissue. This technique was chosen in order to avoid trauma to the subcutaneous tissue. The areas under study were located at the bolar head and at the lateral malleolus. Both legs were investigated.

A catheter was placed in the epidural space corresponding to the third lumbar vertebra.

Blood flow in subcutaneous tissue was measured consecutively with the legs placed horizontally lower in such a way that the proximal and distal depots were lowered 30 and 40 cm below the mid-axillary line respectively and then returned to the horizontal position. Hereafter the legs were elevated so that the depots were raised 30 cm above the mid-axillary line. Finally the blood flow was measured with the legs placed horizontally. Each measurement lasted about 5 min.

Marcain, 0.5%, without adrenaline, was injected into the epidural space through the catheter. The volume was 1.5 ml per segment. The blockade was extended to involve the 6th to 8th thoracic segments. Increase in skin temperature measured on the big toes (Roo *et al.* 1973), disappearance of the sympathetic galvanic reflex (Deos *et al.* 1963), and abolished sweat production, tested by the ninhydrin test (Damen *et al.* 1960), were taken as evidence of an effective sympathetic blockade. After termination of the investigation the "sweat test" was negative. In order to avoid a decrease in arterial blood pressure, 500 ml isotonic saline were infused before the blockade was started. Arterial blood pressure was measured and remained constant throughout the investigation.

After the sympathetic blockade had been achieved, measurements of blood flow in the same areas were repeated as described above. The plasma concentration of adrenaline and nor-adrenaline was measured before and after the sympathetic blockade. Samples of 20 ml blood were taken from a superficial vein for determination of these concentrations according to Valori *et al.* (1973), using an Aminco-Bowman spectrofluorometer (Type B 223-62155).

### Bilateral sympathectomy

Two patients suffering from manual hyperhidrosis were examined before and 24 h after a partial bilateral sympathectomy was performed. Blood flow in subcutaneous tissue in the distal part of the forearm was measured with the investigated area placed at reference level (jugular notch) and lowered 30 and 40 cm below the jugular notch.

A section of the sympathetic chain with one ganglion above and one below the third rib was removed. After the operation, "sweat test" was negative.

### Calculations

Blood flow was measured by means of the <sup>133</sup>Xe washout technique (Sejrsen 1971).

The perfusion coefficient,  $f$ , was calculated from the Kety equation

$$f = k \lambda / 100 (\text{ml}/100 \text{ g min})$$

where  $k$  denotes the rate constant of the washout function ( $\text{min}^{-1}$ ) and  $\lambda$  the adipose tissue to blood partition coefficient ( $\text{ml/g}$ ) (Kety 1951). In the calculations,  $\lambda$  value of 10 was used (Sejrsen 1971). The ad-

on of vascular smooth muscles is due to a local mechanism. When the leg is elevated, o-arteriolar reflex mechanism is not likely because venous pressure remains constant. Therefore, the autoregulation of blood flow demonstrated during elevation is probably due to intrinsic mechanism. A myogenic response of vascular smooth muscles to changes in intraluminal pressure, "myogenic theory" (Bayliss, 1902) and/or liberation by the tissue of vasodilating metabolites, "metabolic theory" (Anrep 1912, Hilton 1971) could be responsible for this intrinsic mechanism. The vasoconstrictor response to increase in transmural pressure might be due to a local myogenic mechanism or perhaps a local reflex mechanism.

The local vasoconstrictor response might be connected with regulation of capillary pressure. Mellander *et al.* (1964) found that capillary filtration coefficient decreased in the feet and arms when they were placed in a dependent position. This finding indicates that the vasoconstrictor response elicited when transmural pressure increases will counteract an increase in capillary pressure and hence an increase in capillary filtration rate (edema protective factor).

The authors wish to express their gratitude to Dr D. Bernevalde and Dr J. E. P. Pedersen, Dept. of Anatomy, Nykøbing FL Hospital, and to Dr W. Passaia, Institute of Medical Physiology B, Copenhagen University for valuable help.

## References

- BEYER, G. On local vascular reactions and their interpretation. *J. Physiol. (Lond.)* 1921 43 313-377.
- BLAIR, W. M., On the local reactions of the arterial wall to changes of internal pressure. *J. Physiol. (Lond.)* 1902 28 220-231.
- CHAMBERLAIN, P. and J. GROSSMAN, Effect of Changes in Limb Position on Peripheral Blood Flow. *Circulation* 1953, 7 478-482.
- CLARKE, G. D., R. ZILBE, S. E. ENFISSE, D. T. MARSH and E. BRAUNWALD, The Role of Skin and Muscle Arteries Vessels in Reflexes Mediated by the Baroreceptor System. *J. clin. Invest.* 1970 49 225-231.
- COPELAND, A. C. and E. ROBINSON, Effects of Raised Venous Pressure in the Circulation of the Isolated Reflex Rabbit Ear. *Amer. J. Physiol.* 1954 125 465-470.
- DE MEYER, F. G. and R. W. VICTOR, Sympathetic Block Persistence After Spinal or Epidural Anesthesia. *J. Neurosurg.* 1963, 18 285-297.
- JONES, W. K.-E., HAGMAR, A. HOWELL and B. G. WALLIN, Microcirculation Affecting Sympathetic Outflow in Human Skin Nerves. *Acta physiol. scand.* 1972 94 177-186.
- LEWIS, K. G., S. ENGLISH and A. WILKINSON, Nephrydium Test—An Objective Method for Testing Local Anesthetic Drugs. *Acta anaesth. scand.* 1960, 4 189-198.
- LOWE, J. Intravascular pressure as a factor regulating the tone of the small vessels. *Acta physiol. scand.* 1949 17 299-310.
- MILLER, P. and A. C. BOYTON, Local Postural Vasomotor Reflexes Arising from the Limb Veins. *Circulation* 1953 7 23-39.
- POPE, F. J. and J. B. SCOTT, Effects of Flow Rate, Venous Pressure, Metabolites, and Oxygen upon Resistance to Blood Flow Through the Dog Forelimb. *Circulation* 1964, XIV XV Suppl. 1 49-59.
- WILEY, H. G., R. O. SACRA and M. J. STRECHER JR., Reflex responsiveness of vascular bed of dog subcutaneous adipose tissue. *Amer. J. Physiol.* 1971 220 993-999.
- FRANKLIN, O., S. L. NEILSEN and W. FAABORG, Autoregulation of Blood Flow in Human Adipose Tissue. *Acta physiol. scand.* 1973 89 533-537.
- FRANKLIN, P. C. and K. M. HAMMOND, Effect of arterial pressure on arterial and venous resistance of isolated forearm. *J. appl. Physiol.* 1962 17 503-508.
- FRANKLIN, P. C. Autoregulatory responses of cat mesenteric arterioles measured in vivo. *Circulation* 1964 22 199-212.
- HILL, E. S. Theory and applications of the exchange of inert gas at the lungs and tissues. *Pharmacol. Rev.* 1951, 3 1-41.

The average concentration in plasma of nor-adrenaline was 280 ng/l plasma (range 492) before and 279 ng/l (range 46–424) after sympathetic blockade. Normal range, ng/l  $p > 0.8$

When the areas under study were lowered 30 and 40 cm below mid-axillary line, flow in subcutaneous tissue decreased to 73 and 57 per cent, respectively before ( $p < 0.001$ ) and to 57 and 63 per cent after the blockade ( $p < 0.001$ ). There was no significant difference between this vasoconstrictor response to increase in transmural pressure obtained before and after the blockade ( $p > 0.8$ ). During 30 cm of elevation, blood flow remained constant before and after the blockade ( $p > 0.5$  and  $p > 0.2$  respectively). The difference in results obtained before and after the blockade was insignificant ( $p > 0.1$ ).

*Bilateral sympathectomy* 24 h after the operation, blood flow at reference level increased about 100 per cent. When the area under study was lowered, blood flow decreased to 36 and 59 per cent ( $p < 0.005$ ) in patient No. 1 and 2, respectively before and to 45 and 59 per cent ( $p < 0.01$ ) respectively after the operation.

### Discussion

When the leg is elevated perfusion pressure declines as venous pressure remains constant. The finding of a constant blood flow under these circumstances indicates a decreased vascular resistance.

When the leg is lowered, perfusion pressure remains constant, but transmural pressure is increased. A finding of a constant blood flow in this case indicates that vascular resistance remains constant, and consequently that the smooth muscles in the walls of the vessels increased their force of contraction just opposing the tendency of passive distension of vessels due to the increase in transmural pressure. A decrease in blood flow indicates an increase in vascular resistance. Such changes in vascular smooth muscle activity may be due to

- 1) *Reflex changes* in sympathetic vasoconstrictor activity due to changes in blood pressure of the central veins and the heart (low pressure baroreceptors) (Roddie *et al* 1958, *et al* 1972, Rowell *et al* 1973)
- 2) *Spinal reflex mechanisms* where e.g. distension of the veins in the extremity might cause arteriolar constriction.
- 3) *Local nervous mechanisms* (Haddy and Scott 1964, Gaskell and Burton 1953).
- 4) *Intrinsic vascular adjustments* to changes in transmural pressure (Bayliss 1902, Fink 1949, Johnson and Hanson 1962, Johnson 1968).

Blood flow in subcutaneous adipose tissue increased during sympathetic blockade. This indicates that sympathetic discharge contributes to the maintenance of the tone of the vessels. The magnitude may be underestimated, because the concentration of adrenaline in plasma increased.

Central sympathetic blockade did not alter the effect of changes in local orthostatic pressure on blood flow in subcutaneous tissue. These findings are in agreement with Constfield (1953) and Gaskell and Burton (1953). When the limb was lowered blood flow decreased significantly 24 h after sympathectomy which indicates that the changes in

## Affinity of Noradrenaline and Dopamine for Neural $\alpha$ Receptors Mediating Negative Feedback Control of Noradrenaline Secretion in Human Vasoconstrictor Nerves

By

LEONART STJÄRNE and JAN BRUNDRÖM

Received 6 March 1975

---

### Abstract

---

Stjärne, L. and J. Brundröm. *Affinity of noradrenaline and dopamine for neural  $\alpha$ -receptors mediating negative feedback control of noradrenaline secretion in human vasoconstrictor nerves.* Acta physiol. scand. 1975. 95. 89-94.

I superfused field stimulated biopsy specimens of human peripheral arteries and veins, preincubated 4-(-)-noradrenaline (NA) to label the neural stores of NA, were used to study the potency of dopamine (DA) and of NA as triggers of  $\alpha$ -adrenoceptor mediated negative feedback control of sympathetic transmitter secretion, evoked by stimulation with trains of 300 shocks at 1 Hz. In this preparation it is found to be only slightly less potent than NA in depressing both the secretion of  $^3\text{H}$ -NA, and the time response, evoked by nerve stimulation. DA depressed the contraction evoked by exogenous NA well, but to very much smaller extent. On the other hand, DA was very weak against the spasm of the smooth muscle; nearly 1 000 times higher concentrations of DA were required to inhibit spasm evoked by exogenous NA. The results show that the neural  $\alpha$ -receptor function involved in that of NA secretion differs considerably from the  $\alpha$ -receptors of e.g. smooth muscle, with respect to DA. It seems possible that the observed depressing effect of DA on NA secretion may be of neurological and clinical interest. It may at least in part explain the vasodilating effect of DA infusions.

---

It is by now considerable evidence that the secretion of noradrenaline (NA) from sympathetic nerves, in many tissues and species, is subject to local negative feedback control, initiated by neural adrenoceptors (Farnbo and Hamberger 1971, Kjekshus and Puig 1971, Enro et al. 1972, Stjärne 1972), capable of sensing the perineuronal concentration of NA, and of mediating restriction of further secretion of sympathetic transmitter. This presynaptic receptor function has been reported to be excited by  $\alpha$ -agonists and blocked by  $\alpha$ -antagonists, and thus to be more closely similar to  $\alpha$ - than to  $\beta$ -receptors of e.g. smooth muscle. Recent observations in isolated guinea-pig vas deferens indicate that the neural receptors in this tissue and species are more sensitive to adrenaline than to NA, while they are highly insensitive to dopamine (DA) (Stjärne 1975). There may exist tissue and/or species differences in this respect, DA has been reported to be about as potent as NA as inhibitor



- MELLANDER, S., B. ÖZERG and H. ODELMAN, Vascular adjustments in increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer. *Acta physiol. scand.* 1964 **1**: 34-48.
- MOLLEY, J. G. A Reduction in Some Vasodilator Responses in Free-standing Man. *Cerebrov. E.* 1969 **3**: 14-21.
- NGAI, S. H., S. ROSELL and L. WALLENBERG, Nervous Regulation of Blood Flow in the Subcutaneous Adipose Tissue of Dogs. *Acta physiol. scand.* 1966 **68**: 397-403.
- NIELSEN, S. L. Adipose tissue blood flow determined by the washout of locally injected  $^{133}\text{Xe}$  in man. *J. clin. Lab. Invest.* 1972 **29**: 31-36.
- ÖZERG, B. and S. ROSELL, Sympathetic Control of Consecutive Vascular Sections in Canine Subcutaneous Adipose Tissue. *Acta physiol. scand.* 1967 **71**: 47-56.
- PAARKE, W. B. and O. HENRIKSEN, Influence of orthostatic blood pressure changes on blood flow in cutaneous tissue of the crus. *Acta physiol. scand.* 1973. *Suppl.* 596: 145.
- RODDIE, J. C. and J. T. SHEPHERD, The Reflex Nervous Control of Human Skeletal Blood Vessels. *Clin. Sci.* 1956 **15**: 433-440.
- RODDIE, J. C., J. T. SHEPHERD and R. F. WIGLIM, Reflex Changes in Human Skeletal Muscle Blood Flow Associated with Intrathoracic Pressure Changes. *Circulation Res.* 1958 **1**: 232-238.
- ROE, F. C. and F. L. COHN, Sympathetic Blockade During Spinal Anaesthesia. *Surg. Gynec. Obstet.* 1972 **136**: 265-268.
- ROSENBERG, E., Local Character of the Veno-Vasomotor Reflex. *Amer. J. Physiol.* 1956 **183**: 472-475.
- ROWELL, L. B., C. R. WYSE and G. L. BAEMOELMANN, Sustained human skin and muscle vasoconstriction with reduced baroreceptor activity. *J. appl. Physiol.* 1973 **35**: 639-643.
- SJURSEN, P. *Assessment of cutaneous blood flow by freely diffusible radioactive isotopes*. Thesis, Copenhagen 1971. Danish Medical Bulletin 1971. *Suppl.* 18.
- VALORI, C., C. A. BRUNORI, V. RENZI and L. CARLA, Improved procedure for formation of epinephrine and nor-epinephrine fluorophores by the trihydroxyindole reaction. *Analyt. Biochem.* 1970 **33**: 155.
- ZOLLER, R. P., A. L. MARK, F. M. ARBOOD, P. G. SCHMIDT and D. D. HEISTAD, The Role of Low Pressure Baroreceptors in Reflex Vasoconstrictor Responses in Man. *J. clin. Invest.* 1972 **51**: 2967-2972.

a prior to the next stimulation, the infusion was stopped 3 min after the end of the stimulation period. In presence of  $0.6 \mu\text{M}$  desipramine and  $10 \mu\text{M}$  normetanephrine, the infused catecholamines did not show the resting effect of NE. Each catecholamine infusion period was terminated by emptying the chamber washing the preparation twice, before superfusion was continued. When the contraction in response to exogenous catecholamine was determined, these were added to the mixing chamber at 6 min intervals, figures given under Results represent their final concentration. When the contraction reached its peak, pen of the Grass polygraph was lifted and the chamber washed twice before the next stimulation. Statistical evaluation of the results was by Student's  $t$ -test. The figures given in the paper represent means  $\pm$  S.E.

## Results

### *chemical effect of nerve stimulation*

Stimulation with 300 shocks at 1 Hz frequently slightly reduced the longitudinal tension of the artery but caused in most preparations a distinct rise in longitudinal tension of the vein. However the rise in tension was only small, and maximum amplification on the Grass polygraph had to be used to visualize it: at this sensitivity level the baseline of the polygraph was no longer horizontal, but drifted gradually upwards. Moreover the preparation showed considerable fatigue: on repeated stimulation the contractile responses declined fairly rapidly (Fig. 1).

### *NA secretion evoked by nerve stimulation*

On stimulation with 300 shocks at 1 Hz, during the initial control period,  $\Delta t$  per shock had an average value of  $(4.50 \pm 0.27) \cdot 10^{-6}$  ( $n = 22$ ). Due to certain variability in the control value of  $\Delta t$ , from one experiment to the other the relative rather than the absolute values are used for calculation of the observed pharmacological effects. The figures for  $\Delta t$  in each experiment were normalized in relation to the average obtained during the first two control stimulations in that same experiment, which was given the value of 1 relative unit.

### *Effect of DA and NA on $\Delta t$*

In 5 expts. DA was infused before NA (Fig. 1), and in 7 the order was reversed. DA  $0.2$  and  $1 \mu\text{M}$  caused dose-dependent and reversible depression of  $\Delta t$ , to  $69.7 \pm 4.0\%$  and  $48.7 \pm 4.2\%$  of the control level, respectively. NA was somewhat more potent in this respect, at  $0.2$  and  $1 \mu\text{M}$  concentration it depressed  $\Delta t$  to  $64.7 \pm 3.6\%$  and  $36.1 \pm 3.6\%$  of the controls. The difference in potency was not significant ( $0.05 > p > 0.01$ ). The average  $\text{IC}_{50}$  (the concentration required to depress  $\Delta t$  to one half) for DA was  $0.90 \mu\text{M}$  while that for NA was  $0.45 \mu\text{M}$ .

### *Effects of DA and NA on mechanical responses*

At  $0.1$  and  $1 \mu\text{M}$  concentration DA did not alter the longitudinal tension of the preparations. However it markedly dose-dependently and reversibly depressed the contractions evoked by nerve stimulation (Fig. 1). NA enhanced the resting longitudinal tension at  $0.2 \mu\text{M}$  only moderately but at  $1 \mu\text{M}$  very markedly (Fig. 1). At  $0.2 \mu\text{M}$  NA the superimposed contraction evoked by nerve stimulation was usually moderately depressed, and at  $1 \mu\text{M}$  NA strongly so (Fig. 1). In many cases the restitution of the contractile response after washing was very incomplete (Fig. 1).

of sympathetic neurotransmitter secretion, in cat spleen and nictitating membrane (Lax 1973).

In a recent study of isolated superfused field stimulated biopsy specimens of human blood vessels, it was found that vasoconstrictor nerves in man also seem to possess an  $\alpha$ -adrenoceptor function, capable of triggering negative feedback control of the secretion of DA (Stjärne and Gripe 1973). The present paper reports results from further studies of this preparation, with the aim to compare the potency of DA with that of NA, as triggers of these, presumably neural, adrenoceptors.

## Material and Methods

The biopsy specimens were taken from 9 normotensive female subjects, aged 29 to 66 years, 8 of the were undergoing gynecological or obstetric surgery (3 cases of Caesarian section) and 1 as support for cholelithiasis. Surgery was performed under various forms of routine general anaesthesia, in some cases with halothane/oxygen/nitrous oxide/cecurtine. Premedication always included atropine 0.5 mg in addition to 5 mg promethazine 25 mg and pethidine 25 to 75 mg s.c. After Caesarian section was performed, oxytocin (Syntocoon®) 2 IU and sometimes Methergine (Sandoz) 0.2 mg were administered by injection into the myometrium.

The biopsy specimens consisted of 5–6 cm pieces of arteries and veins from the omentum, of a caliber of about 1–1.5 mm, excised between ligatures. The tissue was immediately transferred to Tyrode solution (NaCl 0.8%, KCl 0.02%, CaCl<sub>2</sub> 0.02%, MgCl<sub>2</sub> 0.01%, N HCO<sub>3</sub> 0.1%, NaH<sub>2</sub>PO<sub>4</sub> 0.005%, glucose 0.1%, sodium ascorbate 20 µg/ml and atropine sulphate 1 µg/ml), prebubbled with carbogen gas (65% carbon dioxide in oxygen), for transportation to the laboratory. During the subsequent dissection to remove the inert tissue and to separate artery from vein care was taken to avoid damage to the peripheral pieces of sympathetic vasoconstrictor nerves.

In order to label the neural stores of NA, the preparation was then incubated with 10 µCi (240 MBq) <sup>3</sup>H-(+)-NA (New England Nuclear Corp.) per ml Tyrode solution, at 35°C for 30 min. After 10 min for one min each the preparation was mounted in a cylindrical chamber 6 mm in diameter and superfused with Tyrode solution, aerated with carbogen gas (final pH 7.4). The temperature in the chamber was maintained at 31°C and the flow rate was 2.5 ml/min. The superfusate was brought into test tubes in fraction collector; collection periods were 4 min. The longitudinal tension in the preparation was measured with Grass force displacement transducer and recorded on a Grass Polygraph; the resting tension was adjusted to 0.5 g.

The <sup>3</sup>H level of one ml aliquots of the superfusate, and of the tissue (at the end of the experiment) extracted with 0.4 M perchloric acid and then diluted ten-fold with distilled water was determined by counting in 10 ml Instagel (Packard Instr.) in an Intertechnique ABAC SL 40 Liquid scintillation spectrometer. Quenching was monitored by internal standards; the variation between samples in this respect was negligible and was thus not corrected for.

The resting efflux of <sup>3</sup>H declined progressively throughout the experiments. The rise in <sup>3</sup>H efflux evoked by electrical field stimulation (1–90 V on a Grass S 44 stimulator; the preparation was mounted between two parallel platinum wire electrodes, 6 mm apart and 40 mm in uninsulated length) with biphasic pulses 1.5 ms in duration, was completely blocked with tetrodotoxin 3 µM and was thus clearly neural in origin. Since practically all of the <sup>3</sup>H remaining in the tissue after washing was intact <sup>3</sup>H NA, and since 0.6 µM desipramine and 10 µM normetanephrine were added to prevent rebinding of NA (Larsen 1971), the evoked fractional rise in total efflux of <sup>3</sup>H ( $\Delta f$ )

$$\Delta f = \frac{\text{Evoked rise in efflux of } ^3\text{H}}{\text{Calculated total } ^3\text{H in tissue at beginning of stimulation}}$$

was used to measure the secretion of <sup>3</sup>H NA from the nerves. Nervous stimulation with trains of 300 shocks at 1 Hz were applied at 16 (or during washes 32) min intervals.  $\Delta f$  resulting from each stimulus train was calculated from the rise in <sup>3</sup>H efflux during three 4-min collection periods, starting from the beginning of the stimulation.

Exogenous catecholamines were dissolved in HCl (final concentration 0.4 µM), and infused starting

Fig. 2. *Left panel:* Typical record showing rise in longitudinal tension of human splanchnic vein, and by addition of DA and of NA to the medium. The figures represent final  $\mu$ M concentrations. *Right panel:* Effect of DA  $1 \mu$ M rise in longitudinal tension of man splanchnic vein, caused by exogenous NA.



there is considerable reason to regard this as evidence for true receptor-mediated depression of the secretion of neurotransmitter and not as an artefact, due to e.g. uptake and preferential secretion of exogenous unlabelled amine (cf. Stjärne 1975). Interestingly the observed  $IC_{50}$  for NA on A1 in human vasoconstrictor nerves,  $0.45 \mu$ M, was about the same as that found in guinea-pig vas deferens, under the same experimental conditions (Stjärne 1975). This is in contrast to the reported  $IC_{50}$  values for NA in e.g. rabbit heart (Starke 1972) or rat spleen (Langer 1973) of about  $0.06 \mu$ M. It is not possible at present to decide whether this discrepancy is due to methodology or to true tissue and/or species differences. However the present results clearly indicate that the sensitivity level of the  $\alpha$ -receptors of human vasoconstrictor nerves is far too low to be affected by even the highest concentrations of NA which can occur in peripheral arterial plasma, under physiological conditions. On the other hand there can be little doubt that perineuronal concentrations of NA high enough to trigger  $\alpha$ -adrenoceptor mediated negative feedback control of NA secretion do occur in the immediate vicinity of actively secreting adrenergic nerve terminals, and particularly in tissues with narrow synaptic clefts (Stjärne 1973 b, 1975, Stjärne and Gripe 1973). The present results show that DA, while being a very weak agonist on the  $\alpha$ -receptors of the smooth muscle of the preparation, was nearly as potent as NA as agonist on the neural  $\alpha$ -receptors. This is in agreement with observations in cat spleen or nicotinic membrane (Langer 1973), but in distinct contrast to observations made in guinea-pig vas deferens, under identical experimental conditions, in this tissue DA was completely without effect on  $^3$ H NA secretion (Stjärne 1975). Thus there can be no doubt that there do exist true tissue and/or species differences with respect to the selectivity for catecholamines of the neural  $\alpha$ -adrenoceptors mediating feedback control of NA secretion.

The contractile response to nerve stimulation, manifested as a rise in longitudinal tension of the veins, was difficult to demonstrate satisfactorily due to its smallness and rapid decline, on repeated stimulation. DA even at  $1 \mu$ M reduced contraction in response to exogenous NA only slightly. Thus the observed marked reduction of the contractions in response to nerve stimulation, induced by exogenous DA and NA, strongly suggests that the simultaneous depression of  $^3$ H NA secretion was indeed a reflection of depression of secretion of total, endogenous sympathetic transmitter.

It is not possible at present to judge whether the observed inhibitory effect of DA on the secretion of NA from sympathetic nerves in man is of physiological importance. However it may well be of clinical interest, it could explain at least in part, the observed vasodilator effects of intravenous administration of DA, in man (Horowitz *et al.* 1963 McDonald *et al.*

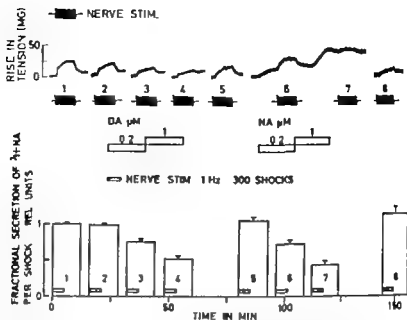


Fig. 1 *Upper panel* Typical record showing effects of DA (dopamine) and of NA (noradrenaline) on rise in longitudinal tension of isolated superfused field stimulated human omental vein, induced by 300 of 300 nerve shocks at 1 Hz. The figures 1-8 refer to stimulation periods in lower panel.

*Lo or panel* Fractional secretion of HNA per shock, in relative units (see Text under Results) nerve stimulation with 300 shocks 1 Hz. Means  $\pm$  S.E. (5 expts. in which DA was infused before N

#### Relative potency of DA and NA on contractile response

DA was very much weaker agonist than NA in evoking contraction. 400-800  $\mu$ M concentrations of DA were required to mimic the contractions induced by 0.2-0.4  $\mu$ M NA (Fig.

#### Effect of DA on contractions evoked by exogenous NA

At 0.2  $\mu$ M concentration DA was without consistent effect on the contractions evoked by exogenous NA. At 1  $\mu$ M DA the responses to NA were very slightly depressed (Fig. 2).

### Discussion

The aim of the present work was to compare the inhibitory effect of NA with that of DA on the secretion of HNA from human vasoconstrictor nerves, evoked by trains of nerve shocks at 1 Hz. The low frequency was chosen because it has been found that efficiency of the receptor-mediated feed-back control of HNA secretions in other preparations is inversely related to nerve stimulation frequency (Stjärne 1973 a). The observed average control value for  $\Delta t$  per shock was in good agreement with that previously reported (Stjärne and Gripe 1973).

Exogenous (-)-NA dose-dependently and reversibly depressed  $\Delta t$  in agreement with previous observations in the same preparation (Stjärne and Gripe 1973). As pointed out in a study of the control of HNA secretion from guinea-pig vas deferens (Stjärne 1973

## The Pulmonary Vasoconstrictor Response to Hypoxia. Effects of Inhibitors of Prostaglandin Biosynthesis

By

J. VAAGØ, L. BJERTNÆS and A. HAUGE

Received 13 March 1975

---

### Abstract

---

J. L. BJERTNÆS and A. HAUGE. *The pulmonary vasoconstrictor response to hypoxic effects of inhibitors of prostaglandin biosynthesis.* Acta physiol. scand. 1975 95 95-101

The purpose of the present work was to determine whether prostaglandins (PGs) synthesized in the vessels mediate the vasoconstrictor response to acute alveolar hypoxia. Isolated and ventilated lungs of rats arterial at 37°C with homologous blood at constant volume, pulmonary inflow and pressure responses to periods of standardized ventilation hypoxia recorded. Indomethacin, sodium meclofenamate and acetylsalicylic acid (all 100 µg/ml), which are potent inhibitors of PG biosynthesis, did not reduce the vasoconstrictor response. Sometimes they even enhanced this response. We conclude that PGs mediate the hypoxia-induced vasoconstriction. We suggest that vasodilatory PGs might act to regulate pulmonary arterial hypertension due to hypoxia.

---

mechanism whereby acute alveolar hypoxia elicits pulmonary vasoconstriction still remains to be properly clarified. Since von Euler and Lijfstrand (1946) demonstrated in cats inhalation of subnormal concentrations of oxygen produced an increase in pulmonary arterial pressure, a number of vasoactive substances have been suggested—and discarded, as possible humoral transmitters of this response. During the last few years several investigators in this field have focused their interest on histamine and other vasoactive agents stored within mast cells situated near small muscular arteries in the lung (Hauge 1968 b, c, and Stanb 1969; Scammano and Carleton 1971; Haas and Bergofsky 1972; Kay *et al.* 1973). The role of histamine has, however, been disputed by some workers (Sjörve and Chis 1973).

In the present work we have focused our attention on another group of substances, namely prostaglandins (PGs). The lungs have a large capacity for synthesis and liberation of PGs secondary to various mechanical and chemical stimuli (Piper and Vane 1971; Piper 1974). Of the various PGs synthesized within the lung, PGF<sub>2α</sub> is a potent constrictor in the pulmonary circulation (Ånggård and Bergström 1963). Furthermore, PGs have been suggested to control ventilation/perfusion ratios in the lung (Lijfstrand 1967; Piper and Vane 1971; Smith 1973). PGs are released from lungs by ventilation (Berry, Edmonds and Wyllie 1971), and PG-like activity of the venous effluent from lungs has been reported to increase

1964 Rosenblum *et al* 1972) This possibility is supported by the recent evidence: Induces vasodilatation in dogs, by blocking neurogenic vasoconstriction (Wille Bogaert 1975)

This work was supported by grants (to L. S.) from the Swedish Medical Research Council (pr. 04X 1027-05), and from Magnus Bergvalls Stiftelse. We thank Mrs Elvör Stjärne, Mrs Ing-Marie and Miss Linda Karlsson for skilful technical assistance.

## References

- ENTRO, M. A., S. Z. LANGER, R. P. ROTHLIN and F. J. E. STEFANO, Role of the alpha-adrenoceptors in regulating noradrenaline overflow by nerve stimulation. *Brit J Pharmacol* 1972, 41 672-683
- FÄRNEBO, L.-O. and B. HAMMERÖR, Drug-induced changes in the release of (<sup>3</sup>H)-noradrenaline from stimulated rat iris. *Brit J Pharmacol* 1971, 43 97-106.
- HOROWITZ, D. S. M. FOX, III and L. I. GOLDBERG, Effects of dopamine in man. *Circulation* 1970, 42 237-243
- IVERSEN, L. L. Role of transmitter uptake mechanism in synaptic neurotransmission. Third Memorial Lecture. *Brit J Pharmacol* 1971, 41 571-591
- KURMEKAR, S. M. and M. PUTO, Effect of flow-stop on noradrenaline release from normal rat spleens treated with cocaine, phentolamine or phenoxybenzamine. *Brit J Pharmacol* 1971, 43 1-10
- LANGER, S. Z. The regulation of transmitter release elicited by nerve stimulation through a feedback mechanism. In *Frontiers in Catecholamine Research* Eds. E. Usdin and S. H. Snyder. New York Toronto Oxford Sydney Braunschweig: Pergamon Press, 1973 543-549
- MCDONALD, R. II Jr, L. I. GOLDBERG, J. L. MCNAV and E. P. TUTTLE, Jr. Effects of dopamine: Augmentation of sodium excretion, glomerular filtration rate, and renal plasma flow. *J Clin Pharmacol* 1964, 43 1116-1124
- ROSENBLUM, R., A. R. TAI and D. LAWSON. Dopamine in man: Cardiovascular hemodynamics in normal patients with heart disease. *J Pharmacol exp. Ther* 1972, 183 256-263
- STARKS, K., Influence of extracellular noradrenaline on the stimulation-evoked secretion of noradrenaline from sympathetic nerves: Evidence for an alpha receptor mediated feedback inhibition of noradrenaline release. *Naunyn-Schmiedeberg's Arch Pharmacol* 1972, 275 11-23
- STJÄRNE, L. Frequency dependence of dual negative feedback control of secretion of sympathetic transmitter in guinea pig vas deferens. *Brit J Pharmacol* 1973 a, 49 353-360.
- STJÄRNE, L., Role of alpha adrenoceptors in prostaglandin E mediated negative feedback control of secretion of noradrenaline from the sympathetic nerves of guinea pig vas deferens. *Proc 1973 b, 4 845-851*
- STJÄRNE, L., Selectivity for catecholamines of presynaptic alpha-receptors involved in feedback control of sympathetic neurotransmitter secretion in guinea pig vas deferens. *Naunyn-Schmiedeberg's Arch Pharmacol* 1975, 288 295-303.
- STJÄRNE, L. and K. GRIF, Prostaglandin-dependent and independent feedback control of noradrenaline secretion in vasoconstrictor nerves of normotensive human subjects: a preliminary report. *Schmiedeberg's Arch Pharmacol* 1973, 280 441-446.
- WILLEMS, J. L. and M. O. BOGAERT. The influence of imipramine on dopamine-induced ganglionic and neurogenic vasodilatation in the dog. *Naunyn-Schmiedeberg's Arch Pharmacol* 1975, 286.

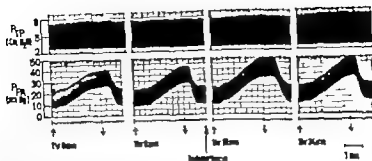


Fig. 1. Premor responses to 3 min periods of ventilation hypoxia between arrows before and after addition of domoic acid (100  $\mu\text{g}/\text{ml}$ ). Constant volume perfusion of isolated rat lungs.  $P_{TP}$  is the pulmonary artery pressure,  $P_{PA}$  is the tracheal pressure.

few tests the response gradually increases until there is a maximum effect for the given depth of hypoxia. Several equal maximum responses can then be obtained before a gradual decline in responsiveness occurs. When finally alveolar hypoxia no longer produces a pressor effect, the pulmonary vascular bed will still respond to vasoactive agents, e.g. kallidin. Since we expected inhibitors of PG-synthesis either to have no effect or to reduce the pressor response to hypoxia, we tested the drugs, in all but one case, in periods when the responses were either increasing or were completely stable. Fig. 1 gives an example of the test procedure. First, two control pressor responses to hypoxia were obtained, whereafter domoic acid (2 mg) was added to the perfusate; then the two following pressor responses were recorded.

Fig. 2 demonstrates in diagrammatic form all the experiments. In the left panel Roman numerals I and II indicate the last two responses ( $\Delta P_{PA}$ ) obtained before drug administration, whereas III and IV indicate the first two responses obtained afterwards. Zero level in

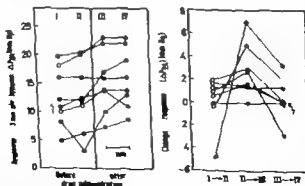


Fig. 2. Left panel demonstrates increases in inflow pressure ( $\Delta P_{PA}$ ) to 3 min periods of ventilation hypoxia in 8 experiments before (○) and after (●) administration of inhibitors of prostaglandin biosynthesis. Numbers to 8 refers to Table I. Here the type of drug given to each individual lung preparation is listed. Right panel demonstrates the difference between one hypoxic pressor response and the preceding response ( $\Delta(\Delta P_{PA})$ ). This difference was increased by the administration of prostaglandin biosynthesis inhibitors, which were given between responses II and III (○).



following deep lung inflations (Said, Kitamura and Vreim 1972, Piper and Vaag 1977 and during hypoxia (Said and Yoshida 1974)

In order to answer the question whether PGs are mediators of the pulmonary pressor response to acute alveolar hypoxia, we have studied the effect of pharmacological inhibition of PG-biosynthesis in an isolated blood-perfused rat lung preparation.

## Methods

**Experimental preparations** Inbred albino rats (local strain), weighing between 180 and 220 g, were anesthetized with pentobarbitone sodium (3–4 mg/100 g b.wt., i.p.) a tracheotomy was performed, and the chest opened during positive pressure ventilation. The trachea, the lungs and the larger intrathoracic vessels were dissected free and heparin was injected (100 I.U. in 0.5 ml saline) into the left ventricle below ligation of the caval veins. Stainless steel cannulas were placed in the pulmonary artery and the left atrium. The cannulas were fastened by a tight ligature around the heart, and the lung preparation was transferred to a humidified, constant-temperature perspex chamber. The lungs were freely suspended in a string tied to the heart ligature and perfused with 20–25 ml heparinized (10 I.U./ml) blood, obtained by puncture of ether-anesthetized donor rats. At the onset of perfusion flow was adjusted so as to give a pulmonary arterial pressure ( $P_{PA}$ ) of about 12–16 mm Hg. Perfusion was begun within 12 min of denervation of the circulation. A Harvard Pulsatile Blood Pump (M 1405) giving constant stroke volume, used  $P_{PA}$  was measured by a Statham P23DB pressure transducer connected to a Sanborn Model 131 amplifier recorder via a Sandborn model 350–1100 B DC preamplifier. Mean pulmonary arterial pressure was calculated as the sum of the diastolic pressure and one-third of the pulse pressure. Controls were obtained with electrical clamping of the pressure gave corresponding results. The venous effluent was drained to an open, double-walled thermostated blood reservoir with a bottom outlet. Since left atrial pressure and volume flow were kept constant, changes in pulmonary vascular resistance were directly reflected changes in mean inflow pressure ( $\Delta P_{PA}$ ). The method was a modification of that described elsewhere (Hauge 1968).

**Ventilation** After one or two gentle inflations of the lungs constant volume positive pressure ventilation was started using a Starlig "Ideal" ventilation pump (C. F. Palmer Ltd.). End-expiratory pressure was kept at about 2 cm  $H_2O$  by means of a water seal. Tracheal pressure was recorded by a diaphragm pressure transducer (Model 270, Hewlett Packard) connected via a preamplifier to the Sanborn recorder. Stroke volume was adjusted so as to give a peak tracheal pressure of about 6 cm  $H_2O$  at the onset. A ventilation frequency of 80 per min was used for all the experiments. The standard gas mixture used in ventilation was 1%  $O_2$ , 5%  $CO_2$  and 74%  $N_2$ . Alveolar hypoxia was induced by ventilating the lungs with a gas mixture of 2%  $O_2$ , 5%  $CO_2$  and 93%  $N_2$  for standardized periods of 3 min with 5 min intervals ("hypoxia tests"). The pH of the perfusate was measured with a Radiometer acid-base analyzer (Mk. PHM 71).

**Drugs.** The following inhibitors of PG synthesis were tested: Indomethacin sodium, meclofenamate and acetylsalicylic acid (Vane 1973, Ferreira and Vane 1974).

**Indomethacin** was prepared as follows: 7.218 g NaHPO<sub>4</sub> was dissolved in 200 ml of sterile water (solution I). 50 ml of this solution was added to 46.1 ml 1 N NaOH. Sterile water was then added up to 50 ml (solution II). 10 ml of solution II were mixed with 2.5 g of Indomethacin. 1 N NaOH was added until Indomethacin was solved at pH about 8.0. Finally solution II was added to a total volume of 100 ml. The prepared Indomethacin solution (25 mg/ml) was stored daily in the refrigerator.

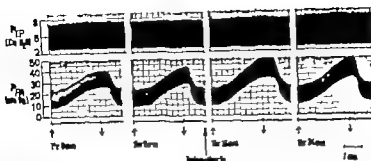
**Sodium meclofenamate** The necessary amount was made up as required with water free of carbon dioxide.

**Acetylsalicylic acid** 70 mg acetylsalicylic acid was solved in 1 ml 96% ethanol, and 19 ml of distilled water was added.

**Kallidin** (Synthetic kallidin, Sandoz A.O.).

## Results

When 3 min periods of alveolar hypoxia are repeated at standardized intervals, the pressor response in isolated rat lungs follows a characteristic pattern (Hauge 1968 a, b). At the beginning of each hypoxic period such "hypoxic tests" produce no or very small pressor responses. After



Prior responses to 3 sets periods of ventilation hypoxia between arrows before and after addition methacin (100  $\mu$ g/ml). Constant volume perfusion of isolated rat lungs.  $P_A$  is the pulmonary artery pressure,  $P_{TP}$  is the tracheal pressure

tests the response gradually increases until there is a maximum effect for the given  $\Delta P_{PA}$  of hypoxia. Several equal maximum responses can then be obtained before a gradual  $\Delta P_{PA}$  in responsiveness occurs. When finally alveolar hypoxia no longer produces a pres-  
fect, the pulmonary vascular bed will still respond to vasoactive agents,  $\alpha$  kallidin.  
as we expected inhibitors of PG-synthesis either to have no effect or to reduce the  
or response to hypoxia, we tested the drugs, in III but one case, in periods when the  
times were either increasing or were completely stable. Fig. 1 gives an example of the  
procedure. First, two control pressor responses to hypoxia were obtained, whereafter  
methacin (2 mg) was added to the perfusate; then the two following pressor responses  
recorded.

Fig. 2 demonstrates in diagrammatic form all the experiments. In the left panel Roman  
erals I and II indicate the first two responses ( $\Delta P_{PA}$ ) obtained before drug administra-  
tion, whereas III and IV indicate the first two responses obtained afterwards. Zero level in

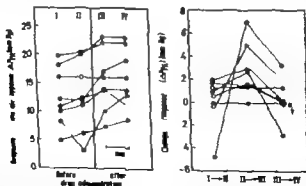


Fig. 2. Left panel demonstrates increase in pulmonary pressure ( $\Delta P_{PA}$ ) to 3 sets periods of ventilation hypoxia (1 sept) before (○) and after (●) administration of inhibitors of prostaglandin biosynthesis. Numbers 1-4 refers to Table I. Here the type of drug given to each individual lung preparation is listed. Right panel demonstrates the difference between one hypoxia pressor response and the preceding response ( $\Delta P_{PA} / \Delta P_{VO}$ ). This difference was increased by the administration of prostaglandin biosynthesis inhibitors such were given between responses II and III (●).

TABLE I

Expt. no	Drug (2 mg)	Volume of perfusate (ml)	Flow (ml/min)	Before drug administration			After drug administration		
				Baseline $P_{PA}$	$\Delta P_{PA}$ I	$\Delta P_{PA}$ II	Baseline $P_{PA}$	$\Delta P_{PA}$ III	$\Delta P_{PA}$ IV
1	Acetylsalicylic acid	25		15.3	10.0	11.0	14.8	13.7	11.1
2	Indomethacin	21		17.7	12.3	12.0	17.3	17.0	21.1
3	Indomethacin	20	14	15.0	18.0	20.0	15.0	23.0	21.1
4	Acetylsalicylic acid	20		11.6	10.7	12.4	12.3	13.7	11.1
5	Indomethacin	20	10	16.0	8.3	3.0	16.0	10.0	11.1
6	Meclofenamate	25		11.6	4.7	6.0	12.0	7.3	11.1
7	Indomethacin	22	4	11.3	19.7	20.3	11.3	22.0	21.1
8	Meclofenamate	20	16	15.0	16.0	16.0	15.0	16.0	16.1

Pulmonary arterial pressure and pressure-changes are given in mm Hg. Peak rise in  $P_{PA}$  during 3 min of ventilation hypoxia.

this panel represents baseline level of  $P_{PA}$  before each individual hypoxic test. With the exception of exp. no. 3 (Table I) the baseline level of  $P_{PA}$  did not change. The right hand part of Fig. 2 demonstrates the change in  $\Delta P_{PA}$  ( $\Delta(\Delta P_{PA})$ ) from I to II, from II to III and from III to IV. Thus, a continuous increase in the response to hypoxia would give positive values of  $\Delta(\Delta P_{PA})$  and if the increase was linear the values would have been identical. A sudden increase in the response, in excess of a steady linear rise, would have increased  $\Delta(\Delta P_{PA})$  compared with the preceding one. Following drug administration this is exactly what happened in the majority of preparations. The response to hypoxia was lifted to a higher plateau. When all the values of  $\Delta(\Delta P_{PA})$  from I to II were regarded as one group and compared with the group of  $\Delta(\Delta P_{PA})$  from II to III, this last group was significantly increased ( $P < 0.05$ , Wilcoxon two-sided test). In no case did inhibitors of PG synthesis cause a reduction in the pressor response to acute hypoxia. Undiminished responses could be elicited as long as 48 min after administration of indomethacin, which is in agreement with the normal response pattern in this preparation.

Table I gives a general overview of preparation parameters such as volume of perfusate, flow and baseline inflow pressure ( $P_{PA}$ ). In addition the table shows which inhibitor of PG synthesis that was given to each individual lung preparation and also the numeric values of pressor responses.

None of the inhibitors of PG-synthesis used had any effect on the vasoconstrictor response to kallidin (100  $\mu$ g) injected intra-arterially.

Neither hypoxic tests nor the administration of inhibitors of PG biosynthesis caused consistent bronchomotor responses, judged from the observation of tracheal pressure.

### Discussion

Inhibition of PG biosynthesis was not experimentally verified in the present study we relied upon the works of other investigators when deciding the dosage of inhibitors be length of the observation periods. The use of nonsteroid anti-inflammatory drugs to test the involvement of PGs in biological systems (Ferreira and Vane 1974 b). and Vane (1969) found that 0.1–0.5  $\mu\text{g/ml}$  of indomethacin and 1–5  $\mu\text{g/ml}$  of aspirin inhibited PG synthesis in isolated guinea-pig lungs perfused with Krebs-Ringer solution. In experiments 2 mg of each agent tested was diluted in about 20 ml of blood, i.e. approximately 100  $\mu\text{g/ml}$ . Since about 90 per cent of indomethacin is bound to plasma-protein (Vane 1973), this is equivalent to an effective dose of this particular drug of some 10  $\mu\text{g}$ , i.e. about 100 times the minimum dose recommended by the above workers. Hermon-Cedro and Vane (1973) have also shown that renal PG-synthesis *in vitro* was inhibited by indomethacin 2 mg/kg. Plasma protein binding of acetyl salicylic acid is 50–80 per cent (Vane 1973). The fraction of meclofenamate which is bound to plasma protein is known (Vane 1973). However when evaluated on a weight basis as well as on a molar basis, this drug is the most potent inhibitor of PG-synthesis of the three used in the present study (Ferreira and Vane 1974 a).

Perfused rat lungs have a considerable capacity to release PGs secondary to a wide variety of stimuli (Bakke and Vane 1974). Whether intrapulmonary synthesis and release of PGs will cause a constrictor or dilator response depends on the balance between the different subgroups of PGs which act on vascular smooth muscle cells.  $\text{PGF}_{2\alpha}$  is a potent pulmonary vasoconstrictor agent (Ånggård and Bergström 1963), whereas  $\text{PGE}_2$  is able to dilate pulmonary resistance vessels, provided there is a certain degree of smooth muscle tone (Hänge *et al.* 1967). The release of PGs from lungs is dependent on a rapidly induced synthesis, since only negligible amounts of PGs are stored in lung tissue (Piper and Vane 1971). Accordingly the present observation that pharmacological blockade of PG synthesis did not diminish the vasoconstrictor response to hypoxia, indicates that vasoconstrictor PGs are not mediators of this response.

An unexpected finding was that nonsteroid, anti-inflammatory drugs sometimes induced moderate potentiation of the vasoconstrictor effect caused by hypoxia. This observation is supported, however by a short recent report from Weir *et al.* (1974), in which it is stated that meclofenamate and indomethacin augment the pulmonary pressor response to hypoxia in intact dogs. As an explanation it is tempting to speculate whether pulmonary arterial hypertension due to hypoxia might induce synthesis and release of some vasodilatory PGs. PG release has been induced by various chemical and mechanical stimuli with distortion of cell membranes (Piper and Vane 1971). The release of some vasodilatory PGs from lungs during severe pulmonary hypertension with subsequent moderation of vascular transmural pressure can be regarded as a functional and advantageous phenomenon modulating the response to hypoxia. Such a postulated mechanism might then be prevented by administration of PG synthesis inhibitors. A secondary release of this type may be the reason why other investigators have observed PG-like biologically active substances in the effluent perfusate from lungs during hypoxia (Seld and Yoshida 1974).

Recently Said and coworkers (1974) reported that the hypoxic pressor response of lungs *in situ* is reduced by aspirin. There are, however, several reasons why this work is difficult to assess. In these experiments aspirin by itself elevated pulmonary vascular resistance (PVR), whereas the PVR-level obtained during hypoxia was unaltered. The reduction in the PVR response to hypoxia was claimed. It was based on calculations in percent of the new PVR base-line levels, assuming a linear system. Furthermore, no test of a pulmonary vasoconstrictor agent was carried out in order to detect unspecific damping of vascular smooth muscle reactivity. Such general and non-specific depression of vascular smooth muscles has been described for rabbit lungs following the administration of nonsteroid anti-inflammatory drugs, such as sodium salicylate and phenylbutazone (Ha et al 1966). Finally, we would like to point out that also Said and coworkers found indomethacin (1 to 10 mg/kg) "sometimes even enhanced" the pulmonary arterial pressure response in cats.

L. B. and J. V. are Research Fellows of the Norwegian Council for Science and the Hamsen's support and additional support, through the Institute of Physiology from the Nansen Foundation and Anders Jahre Foundation for the Promotion of Science together with the generous gifts of indomethacin (Dumex, Copenhagen, Denmark), acetylsalicylic acid (Nyegaard & Co. Oslo, Norway) and sodium salicylate (Parke-Davis, Pontypool, Mon., England) are all gratefully acknowledged.

## References

- ÅMOGLAND E. and S. BERGSTROM, Biological effects of an unsaturated trihydroxy acid (PGF<sub>2α</sub>) from mal swine lung. *Acta physiol. scand.* 1963. 38: 1-12.
- BAKHALE, Y. S. and J. R. VANE, Pharmacokinetic function of the pulmonary circulation. *Pharmacol.* 1974. 54: 1007-1043.
- BERRY P. A., J. F. EDMONDS and J. M. WYLLIE, Release of prostaglandin E<sub>2</sub> and unidentified factor in ventilated lungs. *Brit. J. Surg.* 1971. 58: 189-192.
- EULER, U. S. VON and G. LILJESTRAND, Observations on the pulmonary arterial blood pressure in the cat. *Acta physiol. scand.* 1946. 12: 301-320.
- FERRIERA, S. H. and J. R. VANE, New aspects of the mode of action of nonsteroid anti-inflammatory drugs. *Ann. Rev. Pharmacol.* 1974. 14: 57-73.
- FERRIERA, S. H. and J. R. VANE, Aspirin and prostaglandins in "The prostaglandins" edited by Peter Ramwell, Plenum Press, New York 1974. Vol. 2, pp. 1-47.
- HAAS, F. and E. H. BERKOWSKY, Role of the mast cell in the pulmonary pressor response to hypoxia. *Circ. Res.* 1972. 51: 3154-3164.
- HAUGE, A., Conditions governing the pressor response to ventilation hypoxia in isolated, perfused lungs. *Acta physiol. scand.* 1968. 72: 33-44.
- HAUGE, A., Role of histamine in hypoxic pulmonary hypertension in the rat. I. Blockade and potentiation of endogenous amines, histamine and ATP. *Circulation Res.* 1968. 22: 371-383.
- HAUGE, A., P. K. M. LUNDE and B. A. WAALER, The effect of bradykinin, kallidin and eledren upon the pulmonary vascular bed of an isolated blood-perfused rabbit lung preparation. *Acta physiol. scand.* 1966. 66: 269-277.
- HAUGE, A., P. K. M. LUNDE and B. A. WAALER, Effects of prostaglandin E<sub>2</sub> and adrenaline on the pulmonary vascular resistance (PVR) in isolated rabbit lungs. *Life Sci.* 1967. 6: 673-680.
- HAUGE, A. and N. C. STAUD, Prevention of hypoxic vasoconstriction in cat lung by histamine receptor agent 43/80. *J. appl. Physiol.* 1969. 26: 693-699.
- HEDMAZYNSKA-CZODKO, K. and J. R. VANE, Contribution of intrarenal generation of prostaglandins to the regulation of renal blood flow in the dog. *Circulation Res.* 1973. 33: 428-436.
- KAY J. M., J. C. WAYMIRE and R. T. GROVER, Lung mast cell hyperplasia and pulmonary histamine-forming capacity in hypoxic rats. *Amer. J. Physiol.* 1974. 226: 1783-1787.

13. G., Discussion remarks to Ånggård and Samuelsson: "The metabolism of prostaglandin synthase" in *Proc. 2nd Nobel Symp. Prostaglandins*, edited by S. Bergström and B. Samuelsson. I & Wiksell, Stockholm 1967 pp. 107-108.
14. Release and metabolism of prostaglandins in lung tissue. *Pol. J. Pharmacol. Pharmacy* 1974 2.
15. and J. VANE, Unpublished observations. 1973.
16. and J. R. VANE, Release of additional factors in anaphylaxis and its antagonism by anti-inflammatory drugs. *Nature (Lond.)* 1969 223, 29-37.
17. and J. R. VANE, The release of prostaglandin from lung and other tissues. *Ann. N. Y. Acad. Sci.* 1970, 343-351.
18. KITAMURA and C. VANE, Prostaglandin release from the lung during mechanical ventilation tidal volumes. *J. clin. Invest.* 1972, 51, 83s.
19. and T. YOSHIDA, Release of prostaglandins and other humoral mediators during hypoxic breath-pulmonary edema. *Chest* 1974, 66, Suppl. (part 2), 12A-13A.
20. T. YOSHIDA, S. KITAMURA and C. VANE, Pulmonary alveolar hypoxia: Release of prostaglandin and other humoral mediators. *Science* 1974, 185, 1181-1183.
21. D. and A. J. BROWNE, Histamine-induced pulmonary vasodilatation in the calf: relationship to. *J. appl. Physiol.* 1973, 25, 830-836.
22. P. *The prostaglandins*, edited by P. W. Ramwell. Plenum Press, New York-London, 1973, pp. 203-218.
23. A. and R. A. CARLTON, Prevention of hypoxic pulmonary hypertension by chlorpheniramine. *Physiol.* 1971, 31, 531-535.
24. R., Prostaglandin and Aspirin-like drugs. *Pharmacology and the future of man. Proc. 5th Int. Pharmacology, San Francisco* 1972, vol. 3, pp. 352-378 (Karger, Basel). 1973.
25. K., J. T. RIVERO and R. F. GARCIA, Mefenamic acid and indomethacin suppress the pulmonary response to hypoxia and exogenous prostaglandin  $E_2$ . *The Physiologist* 1974, 17, 355.

Recently Said and coworkers (1974) reported that the hypoxic pressor response of lungs *in situ* is reduced by aspirin. There are, however, several reasons why this paper work is difficult to assess. In these experiments aspirin by itself elevated pulmonary vascular resistance (PVR), whereas the PVR level obtained during hypoxia was unaltered. When reduction in the PVR-response to hypoxia was claimed it was based on calculations in percent of the new PVR base-line levels, assuming a linear system. Furthermore, no tests with a pulmonary vasoconstrictor agent was carried out in order to detect unspecific damping of vascular smooth muscle reactivity. Such general and non-specific depression of vascular smooth muscles has been described for rabbit lungs following the administration of other nonsteroid anti-inflammatory drugs, such as sodium salicylate and phenylbutazone (Haug *et al.* 1966). Finally we would like to point out that also Said and coworkers found that indomethacin (1 to 10 mg/kg) "sometimes even enhanced" the pulmonary arterial pressor response in cats.

L. B. and J. V. are Research Fellows of the Norwegian Council for Science and the Humanities. To support and additional support, through the Institute of Physiology from the Nansen Foundation and the Anders Jahre Foundation for the Promotion of Science together with the generous gifts of indomethacin (Dumex, Copenhagen, Denmark), acetylsalicylic acid (Nyegaard & Co., Oslo, Norway) and sodium methanemate (Parke-Davis, Pontypool, Mon., England) are all gratefully acknowledged.

## References

- ÅNGOÅRD, E. and S. BERGSTROM. Biological effects of an unsaturated trihydroxy acid (PGF<sub>2α</sub>) from the rat twice lung. *Acta physiol. scand.* 1963 58 1-12.
- BAKILLE, Y. S. and J. R. VANE. Pharmacokinetic function of the pulmonary circulation. *Ph. stat. Sc.* 1974 54 1007-1045.
- BERRY, F. A., J. F. EDMONDS and J. M. WYLLIE. Release of prostaglandin E<sub>2</sub> and unidentified factors from ventilated lungs. *Brit. J. Surg.* 1971 58 189-192.
- EULER, U. S. VON and G. LILJESTRAND. Observations on the pulmonary arterial blood pressure in the cat. *Acta physiol. scand.* 1946 12 301-320.
- FERRERA, S. H. and J. R. VANE. New aspects of the mode of action of nonsteroid anti-inflammatory drugs. *Ann. Rev. Pharmacol.* 1974 a. 14 57-73.
- FERRERA, S. H. and J. R. VANE. *Aspirin and prostaglandins*, in "The prostaglandins" edited by Pearcy Ratnoff, Plenum Press, New York 1974 b. Vol. 2, pp. 1-47.
- HAAS, F. and E. H. BIKOROVSKY. Role of the mast cell in the pulmonary pressor response in hypoxia. *Am. J. Physiol.* 1972 221 3154-3162.
- HAUGE, A. Conditions governing the pressor response to ventilation hypoxia in isolated, perfused rat lungs. *Acta physiol. scand.* 1968 a. 72 33-44.
- HAUGE, A. Role of histamine in hypoxic pulmonary hypertension in the rat. 1. Blockade and potentiation of endogenous amines, kinins and ATP. *Circulat. Res.* 1968 b. 22 371-383.
- HAUGE, A., P. K. M. LUNDE and B. A. WAALER. The effect of bradykinin, kallidin and eledoisin upon the pulmonary vascular bed of an isolated blood-perfused rabbit lung preparation. *Acta physiol. scand.* 1966 66 269-277.
- HAUGE, A., P. K. M. LUNDE and B. A. WAALER. Effects of prostaglandin E<sub>2</sub> and adrenaline on the pulmonary vascular resistance (PVR) in isolated rabbit lungs. *Lif. Sci.* 1967 6 673-680.
- HAUGE, A. and N. C. STAUB. Prevention of hypoxic vasoconstriction in cat lung by histamine releasing agent 48/80. *J. appl. Physiol.* 1969 26 693-699.
- HERBAZYNSKA-CEDRO, K. and J. R. VANE. Contribution of intrarenal generation of prostaglandin to the regulation of renal blood flow in the dog. *Circulat. Res.* 1973 33 428-436.
- KAY, J. M., J. C. WAYMIRE and R. T. G. OVER. Lung mast cell hyperplasia and pulmonary histamine-forming capacity in hypoxic rats. *Amer. J. Physiol.* 1974 226 111-116.

der these experimental conditions. This made it of interest to make bioassays in the same species of the antidiuretic activity which could be separated from the urine of goats under normal and stimulated conditions.

### Methods

**Animals.** Eight female goats (b wt. 35 to 40 kg) were used. Two of the animals were used exclusively for bioassays of antidiuretic activity recovered from the urine of the other 6 goats. The animals were routinely confined in metabolism cages at room temperature of  $20 \pm 1^\circ\text{C}$  where they had free access to chaffed hay and water between the experiments. The goats were maintained in positive sodium balance by addition of 6 g NaCl to the 400 g of commercial grain mix they consumed each afternoon.

**Ventricular implantations and infusions.** Four goats were supplied with permanent cannulae in the lateral ventricle near the foramen of Monro. The three-cannulae system used, and the infusion techniques have been described previously (Åkcrand, Andersson and Olsson 1973). Angiotensin II (0.4 mg/kg i.v., type "Ciba") dissolved in hypertonic (0.3 M) NaCl was infused for 30 min periods into the cerebrospinal fluid (CSF) of the lateral ventricle at rates of 10  $\mu\text{l}/\text{min}$ .

**Hydration and dehydration.** Most of the intraventricular infusions were made in the hydrated animal, but water diuresis was fully established. Hydration was accomplished by giving into the rumen by stomach tube 100 ml/kg of  $36^\circ\text{C}$  water 30 min before an intraventricular infusion was started. The two goats used in bioassays of antidiuretic activity were regularly hydrated during the bioassay experiments. When dehydration was used to stimulate ADH secretion, the animals were not given any water to drink for 24 hr before their urine was collected for estimation of antidiuretic activity.

**Urine collection and analysis.** Urine was collected in 10, 30, 60 or 120 min samples via retention catheter inserted into the urinary bladder. Ten min samples were routinely taken in the two goats used solely for bioassays. Urine and plasma  $[\text{Na}^+]$  were determined by use of an EEL flame photometer. An Advanced Instruments Inc. evaporator was used for determinations of urine and plasma osmolality. A plasma osmolality around 290 mosm/kg was found during hydration. Hence, this value was used for calculations of the renal  $\text{Ca}_{\text{H}_2\text{O}}$  in bioassays and other experiments performed in hydrated goats.

**Adsorption and separation of antidiuretic activity in the urine.** The method employed for adsorption and separation of ADH from the urine is principally the same as those described previously by Ruck (1967) and by Fridman (1969). A modification introduced in the column purification with ethanol described below.

Immediately after collection the pH of the urine was adjusted to 3 with trichloroacetic acid (TCA). After cooling and filtration the TCA was extracted with cooled ethyl ether and separated. The small amount of glycerol remaining in the aqueous phase was removed with nitrogen gas. The pH was then adjusted to 8 with 2 M  $\text{NH}_4\text{OH}$  and the urine was allowed to pass by its own hydrostatic pressure through a cation column (Amberlite IRC 50, XE-64). The diameter of the column was 10 mm and its height 40 mm. After passage of the urine the column was washed twice with 30 ml of redistilled water. To avoid substances which in preliminary experiments sometimes had been found to obscure the bioassays, the resin column was purified with 10 ml of 50% ethanol. Finally ADH activity was eluted from the column with 60 ml of 50%  $\text{HAc}$  at  $4^\circ\text{C}$ . The eluate was dried in a rotary evaporator at  $30^\circ\text{C}$ , and the dry material was stored at  $4^\circ\text{C}$  until the time of bioassay. It was then dissolved in 5 or 10 ml of isotonic saline.

Tests are performed in the following way to determine whether the ethanol washing removed any of the ADH activity adsorbed to the resin column. Bioassays were made with aliquots of eight urine samples, where one half of the sample was eluted from columns not treated with ethanol. These bioassays did not reveal any reduction of the ADH activity eluted from ethanol washed columns. Neither did the ethanol washing of the column reduce the recovery of synthetic vasopressin added to urine before passage through the column. This recovery amounted to 80% of the added hormone.

**Tryptan incubation.** To determine ADH activity in control experiments the dissolved eluate was incubated with trypan (Trypan "Novo" 40  $\mu\text{g}/\text{ml}$ ) at pH 7.3 for 5 hr at  $30^\circ\text{C}$ .



## Recovery of ADH Activity in the Urine of Goats under Normal and Stimulated Conditions

By

F LISHAJKO and B ANDERSSON

Received 13 March 1975

---

### Abstract

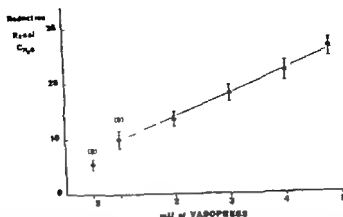
LISHAJKO F and B ANDERSSON *Recovery of ADH activity in urine of goats under normal and stimulated conditions* Acta physiol. scand. 1975 95 102-109

The hydrated goat was used for bioassays of antidiuretic hormone (ADH) activity recovered by column separation from the urine of other animals of the same species. A methodological innovation, the separation procedure was ethanol purification of the column before elution. In this manner effects, which sometimes obscured the bioassays were eliminated without loss of ADH activity. With the basic method employed, it was possible to determine to the nearest 0.5 of a mU the ADH activity of urine samples, provided they contained between 0-5 mU of ADH. When arginine vasopressin was infused intravenously into hydrated animals, slightly more than 10% of its antidiuretic activity was recovered in the urine. In the water replete goat the ADH activity found in the urine was of the order of 1 mU per liter secretion, indicating a neurohypophyseal ADH release of approximately  $5 \mu\text{U/kg min}$ . After 48 h of dehydration in an environmental temperature of  $20^\circ\text{C}$  the renal ADH secretion increased to about 8 mU/l, signifying an 8-fold increase of ADH secretion over basic, water replete secretion of ADH. ADH secretion of the same high order was induced in hydrated animals by the infusion of angiotensin II together with hypertonic NaCl into the lateral cerebral ventricle.

---

Over the past several years goats have been used extensively for studies of the central control of water balance (cf. Andersson and Olsson 1973). In this research the release of antidiuretic hormone (ADH) in response to intracarotid and intraventricular infusions of hypertonic NaCl and angiotensin II has been investigated in the hydrated animal. Evidence that the inhibition of the water diuresis elicited by these stimuli is in fact due to release of ADH has been obtained indirectly. It has been shown that the intracarotid and intraventricular infusions of hypertonic NaCl and angiotensin II no longer elicit an inhibition of the water diuresis when diabetes insipidus has been induced by radio-frequency lesion in the median eminence which interrupts the hypothalamo-neurohypophyseal connections.

The infusion into the third or lateral cerebral ventricle of hypertonic NaCl together with angiotensin II appears to be a very potent stimulus for ADH release in the goat, judged from the long-lasting, profound negative renal free water clearance ( $C_{H_2O}$ ) which develops in the hydrated animal in response to such infusions (Andersson *et al.* 1972). However, no attempt has previously been made to quantitate the amount of ADH which is released



2. Dose-response curve in one of the bioassay animals to graded amounts of synthetic arginine pressin. See "Methods" for explanation of the way in which the significances of the responses were tested. Figures in brackets—Number of tests which each single dose of vasopressin. Vertical bars mean SEM.

### B. Recovery of antidiuretic activity in the urine

#### *Intravenously infused synthetic arginine vasopressin*

two goats (hydrated to inhibit their endogenous ADH secretion) intravenous infusions synthetic arginine vasopressin were made at a rate of 1 and 3 mU/min for 30 min. The urine of these animals was collected during the infusions and as long as the vasopressin forced inhibition of their water diuresis lasted. The urine was eluted and bioassays were performed. In the urine from the goat which received in total 30 mU of vasopressin an antidiuretic activity corresponding to 3 mU was recovered. The recovery in the second animal, which had received in total 90 mU of vasopressin, was 10 mU. Thus, these experiments showed that 10%, or slightly more, of ADH released into the blood might be recovered by the separation and bioassay methods employed.

#### *Water replete and dehydrated goats*

five water replete goats ( $n = 13$ ) 120 min urine samples were collected for elution and bioassay of ADH activity. The mean plasma osmolality of these animals was 294 mosm/kg (range 292 to 296) and their mean plasma  $[Na^+]$  was 145 mEq/l (range 142 to 147). The mean total ADH activity recovered in these 120 min samples was 2 mU (range 1 to 3 mU).

In three of these goats 60 min samples were taken for determination of antidiuretic activity also after 24 and 48 h of dehydration. After 48 h of dehydration the mean plasma osmolality was 305 mosm/kg (range 300 to 310) and the mean plasma  $[Na^+]$  150 mEq/l (range 148 to 151). In the 60 min samples taken at 24 h of dehydration the recovered mean ADH activity was 3 mU (range 2.5 to 3.5). The corresponding recovery at 48 h of dehydration was 8 mU (range 6 to 9 mU), indicating an 8-fold increase over basic, water replete ADH secretion after two days of total water lack. When offered water at this stage of dehydration the three goats immediately drank between 2 and 3.5 liters of water.

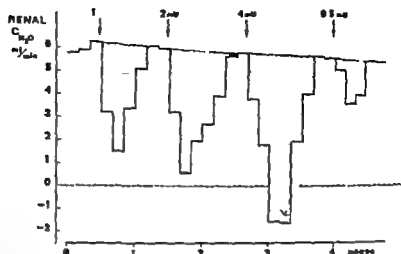


Fig. 1 Antidiuretic responses to graded doses of synthetic arginine vasopressin in one of the two goats used for bioassays of ADH activity recovered from the urine of other goats. Intravenous injections of vasopressin at arrows.  $C_{H_2O}$  = Free water clearance.

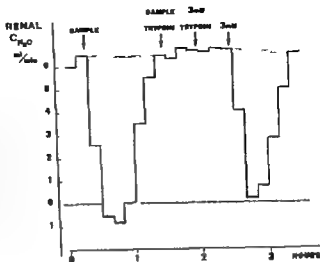
**Bioassay method.** Each animal was used at the most twice a week for bioassay experiments. After initial water loading the water diuresis of the two goats reached a plateau within 80 min (renal  $C_{H_2O}$  +5 to +7 ml/min). Tests were then started of the antidiuretic response to intravenous injections of known ADH (synthetic arginine vasopressin), and of eluates of urine from other goats. Between the periods of inhibition, the  $C_{H_2O}$  approximately returned to the same high, constant level it had at the start of bioassays. This made it possible to make 4 succeeding tests for ADH activity before the estimated loss of water had to be restored by stomach tube to a solid significant decline of  $C_{H_2O}$  between tests.

**Evaluation of bioassay results.** For evaluation of the bioassays the renal  $C_{H_2O}$  was plotted as a histogram on millimeter graph paper. Hence, any temporary inhibition of the water diuresis appeared as a reduction of the surface area occupied by the  $C_{H_2O}$  histogram (Fig. 1). The surface area of this reduction was measured and was compared to the surface reduction induced by the i.v. injection of a known amount of synthetic vasopressin. For simplicity the response to 1 mU of vasopressin was given the figure of 10. In the individual animal, the responses to graded doses of vasopressin calculated in this manner formed a rather linear dose-response curve of constant slope within the range 1 to 3 mU of vasopressin (Fig. 2). Therefore, it was sufficient to make only one or two injections of known amount of vasopressin in each bioassay experiment.

## Results

### A. Sensitivity of the bioassay animals to arginine vasopressin

Repeated experiments were performed in both bioassay goats when only their response to graded doses of arginine vasopressin (0.5 up to 5 mU) were studied. A slight and brief (20 to 30 min) reduction of the water diuresis with remaining positive  $C_{H_2O}$  was obtained by 0.5 mU of vasopressin (Fig. 1), whereas 4 mU invariably induced negative  $C_{H_2O}$  for 20 to 30 min. When calculated as described above, the plotted responses to graded doses of vasopressin followed a rather linear curve in the individual animal (Fig. 2), though some day to day variations in the magnitude of the response to a constant amount of vasopressin were seen the slope of this dose-response curve remained unchanged in experiments performed in the individual animal. Therefore, in subsequent bioassay experiments it was possible to determine to the nearest 0.5 mU the ADH activity of an unknown sample, provided the eluate contained between 0.5 mU of ADH



Trypsin inactivation of activity recovered from the goat subjected to control  $\text{angiotensin}/\text{NaCl}$  stimulation. The sample initially contained an ADH activity (about 4 Arginine vasopressin (AVP) was completely inactivated by incubation.

A recipient was collected for separation and bioassay of ADH activity. An activity of 10 mU was recovered, i.e. slightly more than 10% of the amount of ADH estimated to be infused intravenously.

### C. Trypsin elimination of urinary ADH activity

Incubation with trypsin was employed to obtain further evidence that the antidiuretic activity recovered from the urine exposed true ADH secretion. Intraventricular infusion of angiotensin/NaCl was used to induce antidiuresis in two hydrated goats. The urine collected during the periods of inhibited water diuresis was eluted. Each saline dissolved eluate was divided in two and one half was incubated with trypsin. Subsequent bioassays yielded ADH activities of 3 and 4 mU in the untreated portions of the eluates, whereas no ADH activity remained in the trypsin treated portions. A total disappearance of ADH activity was also obtained when known amounts of synthetic arginine vasopressin were treated with trypsin in the same manner (Fig. 4).

### Discussion

A method initially employed for separation of ADH activity in goats' urine was that described by Ruch (1967) without any modifications. However, injections of eluates obtained by this method occasionally induced sluggishness and a depression of the renal  $\text{CH}_2\text{O}$  of the bioassay animal which apparently was unrelated to the ADH activity of the eluates. Such disturbing side-effects were completely eliminated when ethanol washing of the column was introduced prior to the elution of adsorbed ADH activity. It suggests that the ethanol purification of the column removed toxic substances without releasing significant amounts of ADH adsorbed to the column.

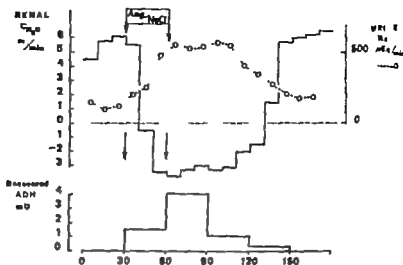


Fig. 3 Above: Antidiuretic and natriuretic responses of a hydrated goat to the infusion of angiotensin II (0.4 ng/kg min) together with hypertonic (0.5 M) NaCl into the lateral cerebral ventricle. Rate of infusion 10  $\mu$ l/min. Below: ADH activity appearing in the urine as effect of the intraventricular angiotensin/NaCl infusion. Note: No detectable ADH activity in the urine during periods of positive renal free water clearance ( $C_{H_2O}$ ).

### 3 Intraventricular angiotensin/NaCl infusions

In four hydrated goats angiotensin II (0.4 ng/kg/min) dissolved in hypertonic (0.5 M) NaCl was infused at 10  $\mu$ l/min into the CSF of the lateral cerebral ventricle for 30 min periods. As expected from previous studies (*cf.* Andersson and Olsson 1973), these infusions had strong antidiuretic and natriuretic effects, and induced thirst. However the animals were allowed to take only occasional small sips of water which revealed that the thirst remained for about half an hour after cessation of the infusions.

In three experiments the urine collected before, during and after the infusion was pooled in 30 min samples and the antidiuretic activity of each sample was eluted and bioassayed. No activity was found in the urine secreted during the 30 min preceding the intraventricular infusion of angiotensin/NaCl. An ADH activity of between 1 and 2 mU was recovered in the 30 min sample taken during the infusion, and the recovered activity rose to a mean of 3.5 mU (range 3 to 4) in the first post infusion 30 min period. It then declined to roughly 1 mU in the second post-infusion period. No, or very little ADH activity was found in the subsequent 30 min sample which was collected when the renal  $C_{H_2O}$  gradually returned to positive values. The results of one of these experiments are presented in Fig. 1.

In four identical experiments the urine was collected from the start of the intraventricular angiotensin/NaCl infusion and for 90 min onwards. These four samples were pooled and eluted. One tenth of the eluate was used for bioassay and showed an ADH activity of 3 mU indicating a total mean recovery of 7.5 mU per experiment. The remaining 90% of the eluate (approximate activity = 27 mU) was then infused intravenously over 30 min to a hydrated goat. The infusion induced negative renal  $C_{H_2O}$  in the recipient which lasted for 110 min. From the start of the infusion and for the entire period of negative  $C_{H_2O}$  the urine

## References

- RO, I. E., I. ANDERSSON and K. OLSSON, A cannula system for frequent infusions into the CSF cerebral ventricles of the goat. *Physiol. Behav.* 1973. 10. 161-162.
- RO, R., I. ERICSSON, O. FREDGAARD, C.-G. KOLMOFF and R. OLSSON, Centrally mediated effects from angiotensin II on arterial blood pressure and fluid balance. *Acta physiol. scand.* 1972. 8-407.
- RO, R. and K. OLSSON, On central control of body fluid homeostasis. *Conditioned Reflex* 1973. -199.
- S, J. P. and R. L. MALVUS, Plasma concentrations of ADH in conscious and anesthetized dogs. *J. Physiol.* 1970 a. 212. 1123-1132.
- S, J. P. and R. L. MALVUS, Stimulation of ADH release by the renin-angiotensin system. *Amer. med.* 1970 b. 218. 1555-1559.
- DE, F. Purification and determination of the antidiuretic substance in human urine. *Acta endocr.* 1969. 42. 31-32.
- DE, H. D. Metabolism of antidiuretic hormones. *Amer. J. Med.* 1967. 42. 713-744.
- DE, H. S. Identification of the antidiuretic substance in human urine. *Acta endocr. (Kbh.)* 1965. 33. 174.
- W. Estimation of antidiuretic hormones in the urine of healthy subjects and patients with inappropriate secretion of vasopressin (Sjögren-Bernier Syndrome). *Acta endocr. (Kbh.)* 1967. 54. 113-121.
- A, S. K. MOTOHARA, H. IMAI and S. OYAMA, Method for the assay of antidiuretic hormones using the antidiuretic test of human plasma. *J. Lab. clin. Med.* 1961. 62. 279-285.
- DE, J. A. JOHNSON and W. W. MOORE, Left atrial pressure, plasma oncolarity and ADH levels in anesthetized rats. *Amer. J. Physiol.* 1969. 217. 1672-1680.

Of importance for the interpretation of the present results is the question whether antidiuretic activity recovered in the urine reflected true secretion of ADH from the neurohypophysis. This seems to have been the case for the following reasons: (1) Eluates of urine collected when the neurohypophyseal release of ADH was inhibited by hydration did not have any antidiuretic effect in the bioassay animals. (2) Dehydration, which accelerates ADH release, markedly increased the antidiuretic activity which could be recovered in the urine. (3) A central stimulus for ADH release (angiotensin in hypertonic NaCl) caused the appearance of large amounts of antidiuretic material in the urine of hydrated goats. (4) Like vasopressin, this antidiuretic material was inactivated by incubation with trypsin. (5) The same degree of urinary recovery was obtained after intravenous infusions of vasopressin, as of antidiuretic material separated from the urine of the goats. Taken together these observations appear to form conclusive evidence that a quantitative measure of renal excretion of ADH was obtained by the methods used in this study.

The fraction of intravenously infused arginine vasopressin which was found to be excreted in the urine of the goats was about 10%. This value falls within the range (5 to 30%) previously found in other mammalian species and man (*cf.* Lauson 1967). Provided the renal excretion of endogenously released ADH is of the same order the present experiments indicated a basic ADH secretion in the water replete, adult goat of about 5  $\mu$ U/4 min. Determinations of renal ADH excretion have indicated a basic ADH secretion of similar magnitude (relative to b.wt) in normal man (Mayer 1960, Ruch 1967).

A 3-fold increase in renal ADH excretion was observed in the goat after 24 h of dehydration at room temperature. After 48 h of dehydration the recovery of ADH activity in the urine was about 8 times higher than in the water replete goat. This relative rise in renal ADH excretion during dehydration in the goat corresponds to the relative increase in plasma ADH level observed during dehydration in man but is somewhat more pronounced than that observed in the dog and the sheep. Dehydration for 16 h was reported to cause a 3-fold increase of the plasma ADH titre in man (Yoshida *et al.* 1963) whereas 24 h of dehydration only doubled the plasma ADH titre of dogs (Bonjour and Malvin 1970a). In sheep dehydrated for 72 h the plasma ADH titre increased by a factor of 4 (Zehr, Johnson and Moore 1969).

It was originally shown by Bonjour and Malvin (1970 b) that angiotensin II stimulates the release of ADH from the neurohypophysis. The intravenous infusion of angiotensin (10 ng/kg min) was found to double the plasma ADH titre in the unanaesthetized dog. Later experiments in the goat have revealed that this effect of angiotensin is Na<sup>+</sup> dependent and centrally mediated (*cf.* Andersson and Olsson 1973). The present study provides a direct confirmation that angiotensin in combination with hypertonic NaCl acts as a very powerful stimulus for ADH release when administered into the cerebrospinal fluid of the lateral cerebral ventricle. In hydrated animals this stimulus induced a urinary excretion of ADH which was of the same magnitude as that observed in the same species after 48 h of dehydration.

was approached by administration of N<sup>2</sup>,2'-O-diibutyl 3',5'-cyclic AMP (DBcAMP), which has been able to mimic almost any known effect related to an increased intracellular level of cAMP. To exclude unspecific adenosine effects, control experiments with equal amounts of 5' AMP (AMP) and adenosine were performed. Furthermore the effects of DBcAMP, AMP and adenosine on liver function as estimated in the splanchnic elimination of ethanol, the hepatic elimination of Indocyanine Green, the bile flow were examined. The effects on splanchnic glucose output, uptake or output of lactate, pyruvate and ketone bodies were also investigated.

## Methods

Male rats weighing 240-410 g and anesthetized with chloralose (50 mg/kg) and Nembutal (30 mg) were used for the experiments. The methods employed were identical with those previously described (Kierup 1973a, b; Kierup and Larsson 1974).

The body temperature was kept close to 38.5°C by gentle heating. Catheters for infusion, blood sampling and/or pressure recordings were placed in femoral artery and vein, hepatic vein and/or portal vein. The intra-vascular pressures were measured by means of condenser manometers placed and calibrated at the arterial level. For collection of bile, a catheter was placed in the choledochum after incision of the cystic duct. A flow probe was placed around the portal vein. When the operation was ended, priming doses of ethanol (6.5 mmol/kg) and Indocyanine Green (ICG) (300 µg/kg) were given in femoral vein, followed by continuous i.v. infusions of ethanol (36 µmol/kg/min), ICG (5 µg/kg/min) and tetrodotoxin (0.20 µmol/kg/min).

The rat was left undisturbed for 90 min. Then blood samples from the femoral artery and hepatic vein were drawn at regular intervals for determination of the concentrations of ethanol, ICG, glucose, lactate (L), pyruvate (P), beta-hydroxybutyrate (HB) and acetoacetate (Ac). The oxygen saturation and hemoglobin concentration in arterial, portal venous and hepatic venous blood were also followed. Bile samples were collected for determination of bile flow and biliary ICG excretion. After a control period of 15 minutes, DBcAMP, cAMP, AMP or adenosine were infused, and blood samples were drawn with the same intervals during another 60 min period.

### Administration of DBcAMP, cAMP, AMP and adenosine

In the preliminary series DBcAMP was infused into the portal vein in doses ranging from 10 to 1000 nmol/kg/min, and cAMP was administered in doses up to 1200 nmol/kg/min. In the rest of the experiments DBcAMP was administered at doses of 85 nmol/kg/min (n=3), 340 nmol/kg/min (n=8), and 50 nmol/kg/min (n=3). In half of these experiments DBcAMP was infused into the portal vein, in the remaining into the femoral vein. The preparations used were commercial products from Boehringer-Ingelheim.

### Calculations

The estimated hepatic blood flow (EHBF) was determined by means of ICG using the Fick principle. The hepatic arterial blood flow was estimated by subtraction of the portal venous flow from the EHBF. The vascular conductances were calculated in the following manner:

$$G_{GC} = G_{IF/EP} \cdot PP \text{ ml kg}^{-1} \text{ min}^{-1} \text{ mmHg}^{-1}$$

$$HAC = HAF/EP \text{ ml kg}^{-1} \text{ min}^{-1} \text{ mmHg}^{-1}$$

$$PVC = G_{IF/PP} \text{ ml kg}^{-1} \text{ min}^{-1} \text{ mmHg}^{-1}$$

(G<sub>GC</sub>, H<sub>AC</sub>, P<sub>VC</sub> conductances in the gastrointestinal area, hepatic artery and intrahepatic portal vein and sinusoids, respectively. G<sub>IF</sub>: gastrointestinal (= portal venous) blood flow. H<sub>AF</sub>: hepatic arterial blood flow. EP: mean arterial blood pressure, PP: portal venous pressure). DBcAMP did not influence the hepatic venous pressure in our experiments, and the measured values corresponded to previous findings in our laboratory (Kierup 1973b). Therefore the hepatic venous pressure was set to zero in the calculation of P<sub>VC</sub>.



## Imitation of Glucagon Effects on Splanchnic Hemodynamics and Liver Function by $N^6,2'$ -O-Dibutryl 3,5-Cyclic AMP (DBcAMP) in Cats

By

NIELS KRARUP, JENS ANKER LARSEN and ANDERS MUNCK

Received 18 March 1975

---

### Abstract

KRARUP N., J. A. LARSEN and A. MUNCK. *Imitation of glucagon effects on splanchnic hemodynamics and liver function by  $N^6,2'$ -O-dibutryl 3,5-cyclic AMP (DBcAMP) in cats* Acta physiol. scand. 1975. 95 110-116

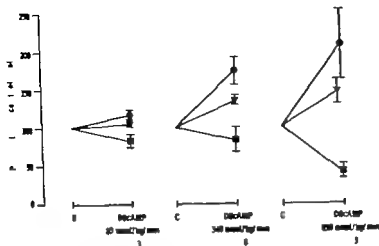
Fasting cats anesthetized with chloralose were used for the experiments. DBcAMP infused at a rate of 340 nmol/kg/min increased the gastrointestinal and intrahepatic portal conductances whereas the hepatic arterial conductance was decreased. The hemodynamic responses to portal and systemic venous administration of DBcAMP were identical. In half of the experiments DBcAMP increased the splanchnic elimination rate and oxygen consumption and in all experiments there was a decrease in the plasma clearance and extraction ratio of Indocyanine Green. No change in bile flow was observed. DBcAMP infused at a rate of 85 nmol/kg/min was without significant effects on either splanchnic hemodynamics or liver metabolism. DBcAMP infused at a rate of 850 nmol/kg/min accentuated the decrease in hepatic arterial conductance but was found to decrease the splanchnic ethanol elimination rate and oxygen consumption. Infusion of cAMP, AMP and adenosine at a rate of 340 nmol/kg/min were without noticeable effects. Based on these results it is concluded that like the metabolic effects also the vascular effects of glucagon are caused by stimulation of specific glucagon receptors which results in an intracellular release of cAMP.

---

Administration of glucagon to cats exerts a marked vasodilatation in the gastrointestinal area which is independent of the changes in hepatic metabolism caused by the hormone (Krarup and Larsen 1974).

It is generally accepted that the metabolic effects of glucagon is caused by liberation of intracellular cyclic AMP (cAMP) according to the second messenger concept (Sutherland and Robinson 1966, Exton *et al.* 1971). It has also been shown that the relaxing action of different agents on isolated vascular smooth muscle is mediated via cAMP (Anderson 1973). It might therefore be expected that the gastrointestinal vasodilatation caused by glucagon is due to an increased level of cAMP in the smooth muscle cells of the gastrointestinal resistance vessels.

The aim of the present study was to examine whether the demonstrated effects of glucagon on hepatosplanchnic hemodynamics could be explained by the second messenger concept.



The effects of different infusion rates of DBcAMP on gastrointestinal, portal venous and hepatic conductances. (I indicates control period, ○ gastrointestinal conductance, ▽ portal venous conductance, □ hepatic arterial conductance.)

#### Splanchnic hemodynamics

Infusion of DBcAMP at a rate of 340 nmol/kg/min caused a small, but not significant increase in arterial blood pressure (from  $147 \pm 8$  to  $158 \pm 9$ ). The portal venous flow increased slightly after the beginning of the DBcAMP infusion and reached a new steady level about 10% higher than the control level in the course of 10 min. In contrast the estimated hepatic arterial flow was slightly but not significantly decreased by DBcAMP. Portal pressure increased to a smaller degree than portal flow. DBcAMP therefore increased the gastrointestinal and intrahepatic portal conductances, whereas hepatic arterial conductance was decreased (see Fig. 2). The hemodynamic responses to portal and systemic venous administration of DBcAMP were identical. When the infusion was stopped, the portal venous flow declined to the control value after 10-15 min.

Infusion of DBcAMP at a rate of 85 nmol/kg/min ( $n=3$ ) had no significant effect on the hemodynamic parameters followed. DBcAMP infused at a rate of 850 nmol/kg/min ( $n=3$ ) caused a decrease in hepatic arterial conductance, whereas no further increase in gastrointestinal and portal venous conductances occurred. The results are illustrated in Fig. 2.

DBcAMP and adenosine infused at a rate of 340 nmol/kg/min caused no significant changes in splanchnic hemodynamics.

#### Liver function and metabolism

In half of the experiments in which DBcAMP was given at a rate of 340 nmol/kg/min the initial elimination rate of ethanol was low and increased 22% (from  $27 \pm 2$  to  $33 \pm 2$   $\mu\text{mol/kg/min}$  ( $p < 0.001$ )) together with a 38% increase in hepatic oxygen consumption (from  $16 \pm 6$  to  $66 \pm 8$   $\mu\text{mol/kg/min}$  ( $p < 0.05$ )). In 3 cats in which the initial elimination rate of ethanol was larger ( $33$   $\mu\text{mol/kg/min}$ ) DBcAMP did not influence the splanchnic ethanol elimination rate and the hepatic oxygen consumption. In none of the experiments was any

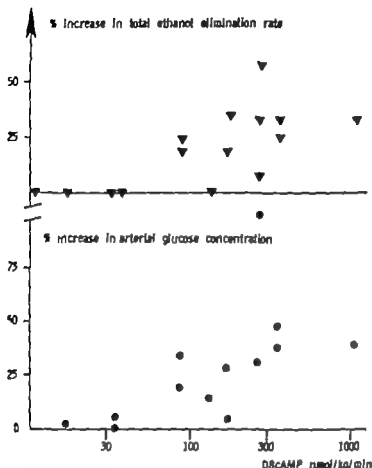


Fig. 1 The effects of various infusion rates of DBcAMP on  $\gamma$ -ethanol elimination rate and arterial glucose concentration.

The total elimination rate of ethanol was calculated as the amount infused per minute corrected for amount disappeared from  $\gamma$  retained in the solvent space set as 65% of the body weight (Larsen 1966). The splanchnic uptake or output of the different substances were calculated from the arterio-venous concentration gradients and the EHBF. The hepatic oxygen consumption was determined as the difference between total splanchnic and gastrointestinal oxygen consumption. The clearance and fraction ratio of ICO was determined by dividing the amount eliminated respectively the arterio-hepatic venous difference by the arterial plasmaconcentration at equilibrium.

#### Statistical procedure

The significance levels were determined by the method of paired comparison using the Student's *t*-test.

### Results

In 5 preliminary expts. with cAMP given at doses up to 1200 nmol/kg/min no hemodynamic or metabolic changes could be demonstrated. In a preliminary series of 15 expts. a dose-response relationship of DBcAMP was examined. The effects of various infusion rates on total ethanol elimination rate and arterial glucose concentration are illustrated in Fig. 1. In 5 expts. in which the infusion rates varied from 130–340 nmol/kg/min, an increase in portal flow of about 50% was observed. Based on these results an infusion rate of 340 nmol/kg/min was chosen for further examination of the effects of DBcAMP.

## Accumulation of Calcium by Retinal outer Segments

By

KARI HEMMONKILÄ

Received 18 March 1975

### Abstract

Hemmonkilä, K. Accumulation of calcium by retinal outer segments. *Acta physiol. scand.* 1975. 95: 117-125.

Preparations of bovine photoreceptors, including intact and lysed outer segments, are used to study kinetics of  $\text{Ca}^{++}$  in order to differentiate between binding and transport. The two preparations accumulate roughly equal amounts of  $\text{Ca}^{++}$  in the presence or absence of ATP. In the absence of ATP no further accumulation occurs. With ATP accumulation continues most rapidly in intact outer segments. The addition of  $\text{Ca}^{++}$ -ionophore (A 23187) to assays containing ATP results in kinetics of accumulation similar to those in the absence of ATP. Thus the initial phase of  $\text{Ca}^{++}$  accumulation is predominantly binding, while that reduced by ATP is due to transport. Accumulation of  $\text{Ca}^{++}$  by outer segments is stimulated by preincubating them with ATP suggesting that ATP enters the structure and acts intracellularly. Illumination decreases binding of  $\text{Ca}^{++}$  to outer segments but does not consistently affect transport. After illumination more  $\text{Ca}^{++}$  is retained by intact than lysed outer segments indicating that outer membrane acts as a barrier to the escape of  $\text{Ca}^{++}$ . Phospholipids and proteins appear to be possible for  $\text{Ca}^{++}$  binding of outer segments as suggested by reduced binding ability after removal of these components. Phosphatidyl ethanolamine is the main phospholipid class of outer segments.

Known ions have been suggested to mediate between absorption of light and hyperpolarization of the cell membrane in retinal photoreceptors (Tomita 1970, Hagins 1972). Evidence is now accumulating in support of the involvement of  $\text{Ca}^{++}$  in light-induced signals.  $\text{Ca}^{++}$  has been reported to be released from outer segments on illumination (Mason *et al.* 1974, Hemmonkilä 1973) and some data suggest that accumulation of  $\text{Ca}^{++}$  by outer segments is regulated by light (Mason *et al.* 1974).

Accumulation of  $\text{Ca}^{++}$  ions by photoreceptors is stimulated by exogenous ATP (Bownds *et al.* 1971, Neufeld *et al.* 1972) but it has not been established whether ATP increases binding or transport of the ion. In this study accumulation of  $\text{Ca}^{++}$  by intact and lysed bovine outer segments is investigated. A calcium ionophore, A23187 (Garwell and Pressman 1972) applied in the separation of binding and transport. Regulation of accumulation of  $\text{Ca}^{++}$  by light is investigated and the  $\text{Ca}^{++}$  binding component is characterized.

## References

- ANDERSON, R. Cyclic AMP as a mediator of the relaxing action of papaverine, nitroglycerine, etc. and hydralazine in intestinal and vascular smooth muscle. *Acta pharmacol. (Kbh.)* 1973, 32, 231.
- DOERS, L. W. and G. A. ROBINSON, Functional biochemistry of beta receptors in the uterus. *Id.* 1968, 27, 352.
- EXTON, J. H. G. A. ROBINSON, E. W. SUTHERLAND and C. R. PARK, Studies of the role of cyclic 3',5'-monophosphate in the hepatic actions of glucagon and catecholamines. *J. Biol. Chem.* 1971, 246, 6166-77.
- HENON, W. F. E. W. SUTHERLAND and TH. POSTERNAK, Effects of derivatives of adenosine 3',5'-phosphate on liver slices and intact animals. *Biochem. biophys. Acta (Amst.)* 1967, 143, 106-13.
- KAUKEK, E. and H. HILZ, Permeation of dibutyryl cAMP into HeLa cells and its conversion to cAMP. *Biochem. biophys. Res. Commun.* 1972, 46, 1011-19.
- KRARUP, N. The effect of noradrenaline and adrenaline on hepatosplanchnic hemodynamics, liver capacity of the liver and hepatic metabolism. *Acta physiol. scand.* 1973 a, 87, 307-19.
- KRARUP, N. The effect of hemorrhage on hepatosplanchnic hemodynamics, liver function and metabolism. *Acta physiol. scand.* 1973 b, 89, 269-77.
- KRARUP, N. and J. A. LARSEN, The effect of glucagon on hepatosplanchnic hemodynamics, liver capacity and metabolism of the liver in cats. *Acta physiol. scand.* 1974, 91, 41-52.
- KRARUP, N., J. A. LARSEN and A. MUNCK, The effects of glucagon and dibutyryl cyclic AMP on hepatosplanchnic metabolism and hemodynamics in cats. *Digestion* 1974, 10, No. 4-5, 361.
- LARSEN, J. A., Elimination of ethanol as a measure of the hepatic blood flow in the cat. *Acta physiol. scand.* 1963, 57, 209-23.
- LARSEN, J. A. and N. KRARUP, Relation between the hepatic elimination rate of ethanol and mitochondrial respiration, Alfred Benzon Symposium VI, Munksgaard Copenhagen 1974, 434-42.
- MEHANAN, L. A. and O. WIELAND, Glucagon-like action of  $N^6,2'$ -O-dibutyryl cyclic 3',5'-AMP on fused rat liver. *Biochem. biophys. Res. Commun.* 1967, 29, 880-85.
- MOORE, P. F., L. C. IORIO and J. M. McMANUS, Relaxation of the guinea-pig tracheal chain prepared by  $N^6,2'$ -O-dibutyryl 3',5'-cyclic adenosine monophosphate. *J. Pharm. Pharmacol.* 1968, 20, 361-2.
- SUTHERLAND, E. W. and G. A. ROBINSON, The role of cyclic 3',5'-AMP in responses to catecholamines and other hormones. *Pharmacol. Rev.* 1966, 18, 145-61.

## Accumulation of Calcium by Retinal outer Segments

By

KARI HEMMENIKI

Received 18 March 1975

### Abstract

Hemmeniki, K. Accumulation of calcium by retinal outer segments. *Acta physiol. scand.* 1975, 95, 117-125

Preparations of bovine photoreceptors, including intact and lysed outer segments, are used to study regulation of  $\text{Ca}^{++}$  in order to differentiate between binding and transport. The two preparations achieve initially roughly equal amounts of  $\text{Ca}^{++}$  in the presence or absence of ATP. In the absence of ATP no further accumulation occurs. With ATP accumulation continues most rapidly in intact outer segments. The addition of  $\text{Ca}^{++}$ -ionophore (A 23187) to assays containing ATP results in kinetics of accumulation similar to those in the absence of ATP. Thus the initial phase of  $\text{Ca}^{++}$  accumulation is predominantly due to binding, while that reduced by ATP is due to transport. Accumulation of  $\text{Ca}^{++}$  by outer segments is stimulated by preloading them with ATP suggesting that ATP enters the structures and acts intracellularly. Dissolution decreases binding of  $\text{Ca}^{++}$  to outer segments but does not considerably affect transport. After dissolution more  $\text{Ca}^{++}$  is retained by intact than lysed outer segments indicating that the membrane acts as a barrier to the escape of  $\text{Ca}^{++}$ . Phospholipids and proteins appear to be suitable for  $\text{Ca}^{++}$  binding of outer segments as suggested by reduced binding ability after removal of these constituents. Phosphatidyl serine/phosphatidyl ethanolamine is the main phospholipid class of outer segments.

Calcium ions have been suggested to mediate between absorption of light and hyperpolarization of the cell membrane in retinal photoreceptors (Tomita 1970, Hagins 1972). Recent evidence is now accumulating in support of the involvement of  $\text{Ca}^{++}$  in light-induced signal transduction.  $\text{Ca}^{++}$  has been reported to be released from outer segments on illumination (Mason *et al.* 1974, Hemmeniki 1975) and some data suggest that accumulation of  $\text{Ca}^{++}$  by outer segments is regulated by light (Mason *et al.* 1974). Accumulation of  $\text{Ca}^{++}$  ions by photoreceptors is stimulated by exogenous ATP (Bownds *et al.* 1971, Neufeld *et al.* 1972) but it has not been established whether ATP increases binding or transport of the ion. In this study accumulation of  $\text{Ca}^{++}$  by intact and lysed bovine outer segments is investigated. A calcium ionophore, A23187 (Cawwell and Pressman 1972) is applied in the separation of binding and transport. Regulation of accumulation of  $\text{Ca}^{++}$  by light is investigated and the  $\text{Ca}^{++}$  binding component is characterized.

## Materials and Methods

### Isolation of outer segments

Bovine eyes were removed immediately after slaughter and placed in a light-proof container at 4°C for transportation. After about 1 h retinas were dissected under dim red light, washed in 0.25 M and collected in 1.0 M sucrose (0.5 ml/retina at 0°C). The suspension of retinas was shaken by 2–15 s and transferred into a beaker which was covered with a nylon mesh (hole size 0.5 mm); liquid was allowed through the mesh and the contents of the beaker were washed with 0.25 M sucrose (ml/retina); the wash was filtered through the mesh and combined with the first slurry. The mixture was centrifuged at 9000 g for 10 min and the supernatant was decanted. The pellet was resuspended in 1.05 M sucrose (1.5 ml/retina) and transferred into cellulose nitrate tubes. 5 ml of 0.25 M sucrose overlaid on 25 ml of 1.05 M sucrose containing the sample and the gradient was centrifuged in a SW 25 rotor at 63000 g for 30 min. The interfacial layer was collected with a Pasteur pipette, diluted 3 vol. of 0.25 M sucrose and sedimented at 9000 g for 10 min. The pellets contained retinal outer segments. Outer segments were lysed by maintaining in distilled water for 10 min. The extent of lysis was by injecting samples of outer segments (0.1 ml) into 0.12 M ammonium acetate (1 ml) and rapidly measuring the change of absorbance at 640 nm.

### Procedures

$\text{Ca}^{++}$  accumulation was assayed as described recently (Hemminki, 1974) by incubating samples of outer segments (50  $\mu\text{l}$ ) in physiological medium containing 0.12 M NaCl, 10 mM KCl, 1 mM  $\text{MgSO}_4$ , 1 mM  $\text{CaCl}_2$  and 10 mM sodium phosphate buffer pH 7.4.  $^{45}\text{CaCl}_2$  (i.e. 10–40 mCi/mg  $\text{Ca}^{++}$ , Amersham) was used at 0.5  $\mu\text{Ci}$ . Incubations were performed in room light at 37°C. Incubations were terminated by adding 5 ml of 0.1 M Tris-HCl, pH 7.5 and transferring the mixtures onto Millipore filters (0.45  $\mu\text{m}$ ), presoaked in 0.25 M KCl. The membranes were washed with 2–4 ml batches of Tris with acetone and counted in 5 ml of Bray's scintillation fluid. Blanks contained no outer segments. A calcium ionophore A 23187 (Cashwell and Pearson 1972; Foreman *et al.* 1973), was a gift from Eli Lilly.

Purity of outer segments was investigated by assaying for marker enzymes including choline acetyltransferase (E.C. 2.3.1.8), a marker of neuronal membranes, succinate dehydrogenase (E.C. 1.3.99.1), a mitochondrial marker and rotenone-insensitive NADH-cytochrome C oxidoreductase (E.C. 1.6.99.3) a microsomal marker. The enzyme assays are described elsewhere (Hemminki and Suovaniemi 1973).

Phospholipids were extracted by the system of Folch and analysed as described previously (Hemminki 1973). Cholesterol was determined according to Pearson *et al.* (1953).

Electron microscopy was performed according to Sabatini *et al.* (1963).

## Results

### Characterization of outer segments

Intact bovine retinal outer segments were prepared by gradient centrifugation and for preservation of structure they were kept in isotonic or for short times, in hypertonic solutions. Outer segments of this preparation appeared intact and were surrounded by unruptured plasma membranes (Fig. 1). Lysed outer segments (Fig. 2) were prepared by suspending outer segments in water. The fraction composed of vesicularized membranes. The degree of lysis of outer segments was checked by an osmotic test. Outer segments were suspended in ammonium acetate, which passes freely into these structures causing osmotic swelling (Hemminki *et al.* 1971). These results are shown in Table I. Absorbances of the fractions (2.0  $\mu\text{g}$  protein) were 1.26 and 0.03 in intact, and lysed outer segments, respectively. In good agreement with the electron microscope observations.

Purity of outer segments was examined with marker enzymes and spectral ratios (Table II). Markers of neuronal and microsomal membranes showed high purity of outer segments.



Fig 2

1 An electron micrograph of intact bovine retinal outer segments, 6 000.

2 Lysed outer segments, prepared by shearing outer segments in water for over 10 min, 3 900.

The specific activities of these enzymes in the samples were only 7% of the homogenate activities.

No mitochondrial contamination was detected in outer segments. The spectral ratios indicated concentration of rhodopsin by 6.5 to 10-fold which is among the best purifications reported in the literature (Zorn and Futscher 1973, Deenen 1973).

#### Accumulation of calcium by outer segments

The effect of added ATP on the accumulation of calcium was studied with intact and lysed outer segments (Fig. 3). At 1 mM concentration ATP increased the initial accumulation of calcium only slightly. Within 30 min a 4-fold increase was observed with intact outer segments compared to a less than 2-fold increase with lysed outer segments. As lysed outer segments are osmotically inactive, they are likely to lack transport functions. Thus ATP may enhance a transport mechanism for Ca<sup>++</sup> rather than facilitate binding of the ion.

In order to differentiate between binding and transport a calcium ionophore A 23187 (Ashwell and Pressman 1977) was used (Fig. 4). Kinetics of Ca<sup>++</sup> uptake in the presence

TABLE 1. Osmotic properties of intact and lysed bovine outer segments. Samples were mixed with 0.12 M tris-maleic acid buffer and absorbance was monitored at 640 nm

	$\Delta$ absorbance/mg protein
Intact outer segments	1.26
Lysed outer segments	0.03



TABLE II Enzymatic and spectral characterization of lysed retinal outer segments. Enzyme activities expressed in nmol/min/mg protein. Means  $\pm$  S.E. of 4 to 5 determinations.

	A Outer segments	B Homogenate	B/A
Cholinesterase	$2.1 \pm 0.8$	$29.3 \pm 2.2$	14.0
Succinate dehydrogenase	0	$49.2 \pm 2.9$	$\infty$
Rotenone-insensitive NADH cytochrome C reductase	$8.0 \pm 2.4$	$124 \pm 10.1$	15.5
A278/A498	$2.24 \pm 0.07$	$14.5 \pm 0.7$	6.5
A400/A498	$0.294 \pm 0.04$	$3.01 \pm 0.07$	10.3

of A 23187 were largely similar to those observed in the absence of ATP (Fig. 3A). Net accumulation of  $\text{Ca}^{++}$  occurred after 1 min. It therefore appeared likely that the uptake was largely due to binding of  $\text{Ca}^{++}$  while the delayed uptake, induced by ATP would be due to transport. If it is assumed that A 23187 makes membranes of outer segments freely permeable to  $\text{Ca}^{++}$  Fig. 4B would then describe the maximal  $\text{Ca}^{++}$  binding capacity of the membranes about 5 nmol  $\text{Ca}^{++}$ /mg protein. It is of interest to refer to Fig. 3A showing that even intact outer segments acquire about 50% of the maximal binding capacity within 1 min. This indicates ready accessibility of a number of  $\text{Ca}^{++}$  binding sites.

Accumulation of  $\text{Ca}^{++}$  by outer segments was studied at 2 different  $\text{Ca}^{++}$  concentrations 1 mM and  $4 \times 10^{-6}$  M as shown in Fig. 5. At low  $\text{Ca}^{++}$  concentrations the kinetics were largely similar to those at the high concentration of  $\text{Ca}^{++}$ .

Stimulation of  $\text{Ca}^{++}$  uptake by outer segments, described in Fig. 3 was suggested to reflect stimulation of transport. It was therefore of interest to show whether ATP entered outer segments. Outer segments were incubated in the presence of ATP at  $0^\circ\text{C}$  for 30 min and assay for  $\text{Ca}^{++}$  uptake was performed. If the uptake was stimulated as compared to the control samples, where ATP was added immediately before the assay this would then suggest that ATP slowly diffused into outer segment. This appeared to be the case (Table III). Uptake was stimulated by a factor of 1.3 in intact outer segments. The stimulation of transport was possibly even higher since binding, as measured in the presence of the calcium ionophore was somewhat lower in the experimental samples. These data suggest that ATP stimulates transport of  $\text{Ca}^{++}$  into outer segments intracellularly.

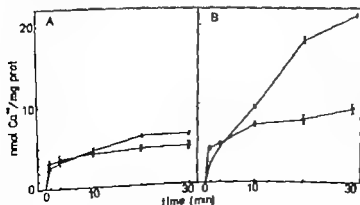
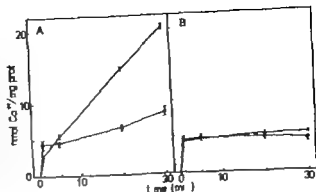
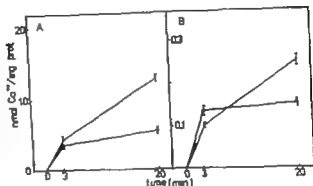


Fig. 3. Effect of ATP on the accumulation of radioactive calcium to retinal outer segments. Outer segments containing about 90% of protein were incubated in physiological medium containing  $\mu\text{Ci } ^{45}\text{CaCl}_2$  in total volume 450  $\mu\text{L}$ . In (B) 1 mM ATP included. (●—) intact outer segments, (○—) lysed outer segments. Means  $\pm$  S.E. of 4 determinations.

4. Effect of A 23187 on the accumulation of radioactive calcium into outer segments. Incubation conditions as described in Fig 3. Assays were performed in the presence of 1 mM ATP (B) control 0.9  $\mu\text{M}$  A 23187 (—●—) or outer segments (—○—). Means  $\pm$  S.E. of 4 experiments.



5. Effect of calcium concentration on the accumulation of radioactive calcium in retinal outer segments. (A) was incubated in physiological medium containing 1  $\mu\text{M}$   $^{45}\text{CaCl}_2$  and 0.5  $\mu\text{M}$   $^{45}\text{CaCl}_2$  (B) in non-radioactive calcium was omitted. The incubation was performed in the presence of 1 mM ATP (—●—) intact outer segments (—○—) lysed outer segments. Means  $\pm$  S.E. of 4 experiments.



6. Effect of illumination on the accumulation of calcium

Light regulation of  $\text{Ca}^{++}$  accumulation by outer segments was investigated by incubating illuminated and unilluminated preparations in the presence of radioactive  $\text{Ca}^{++}$  (Table IV). Incubation conditions were such that mainly binding of  $\text{Ca}^{++}$  was probed, e.g. absence of ATP and presence of A 23187. Illuminated preparations bound consistently less calcium. Since the assays were carried out in conditions suitable to monitor transport, no light regu-

7. Table IV. Accumulation of calcium by outer segments preloaded with ATP. Outer segments were preincubated in physiological medium with or without 2 mM ATP at 0°C in the dark. After 30 min 2 mM ATP was added to the control samples; 0.5  $\mu\text{M}$   $^{45}\text{CaCl}_2$  and as indicated cases 1  $\mu\text{M}$  A 23187 were added. Samples were incubated at 37°C for 10 min and collected on filters. Means  $\pm$  S.E. of 8 determinations. Significant at  $p < 0.01$  in two-tailed Student's  $t$ -test.

	Calcium binding, nmol $\text{Ca}^{++}$ /mg protein	
	Preincubation with ATP	Preincubation without ATP
Intact outer segments	17.4 $\pm$ 0.8	13.6 $\pm$ 0.5
A 23187	4.20 $\pm$ 0.34	7.42 $\pm$ 0.88
Lysed outer segments	6.92 $\pm$ 0.41	6.25 $\pm$ 0.22
A 23187	4.31 $\pm$ 0.29	4.88 $\pm$ 0.32

TABLE II Enzymatic and spectral characterization of lysed retinal outer segments. Enzyme activity expressed in nmol/min/mg protein. Means  $\pm$  S.E. of 4 to 5 determinations.

	A Outer segments	B Homogenate	B/A
Cholinesterase	2.1 $\pm$ 0.8	29.3 $\pm$ 2.2	14.0
Succinate dehydrogenase	0	49.2 $\pm$ 2.9	$\infty$
Rotenone-insensitive NADH cytochrome C reductase	8.0 $\pm$ 2.4	124 $\pm$ 10.1	15.5
A278/A498	2.24 $\pm$ 0.07	14.5 $\pm$ 0.7	6.5
A400/A498	0.294 $\pm$ 0.04	3.01 $\pm$ 0.07	10.3

of A 23187 were largely similar to those observed in the absence of ATP (Fig. 3) net accumulation of  $\text{Ca}^{++}$  occurred after 1 min. It therefore appeared likely that the uptake was largely due to binding of  $\text{Ca}^{++}$  while the delayed uptake, induced it would be due to transport. If it is assumed that A 23187 makes membranes of outer segments freely permeable to  $\text{Ca}^{++}$  Fig. 4 B would then describe the maximal  $\text{Ca}^{++}$  capacity of the membranes about 5 nmol  $\text{Ca}^{++}$ /mg protein. It is of interest to refer to Fig. 3 A showing that even intact outer segments acquire about 50% of the maximal capacity within 1 min. This indicates ready accessibility of a number of  $\text{Ca}^{++}$  binding sites.

Accumulation of  $\text{Ca}^{++}$  by outer segments was studied at 2 different  $\text{Ca}^{++}$  concentrations 1 mM and  $4 \times 10^{-6}$  M as shown in Fig. 5. At low  $\text{Ca}^{++}$  concentrations the kinetics were largely similar to those at the high concentration of  $\text{Ca}^{++}$ .

Stimulation of  $\text{Ca}^{++}$  uptake by outer segments, described in Fig. 3, was suggested to be stimulation of transport. It was therefore of interest to show whether ATP enters outer segments. Outer segments were incubated in the presence of ATP at 0°C for 30 min and then an assay for  $\text{Ca}^{++}$  uptake was performed. If the uptake was stimulated as compared to control samples, where ATP was added immediately before the assay, this would then suggest that ATP slowly diffused into outer segment. This appeared to be the case (Table II). Uptake was stimulated by a factor of 1.3 in intact outer segments. The stimulation of transport was possibly even higher since binding, as measured in the presence of the ionophore, was somewhat lower in the experimental samples. These data suggest that ATP stimulates transport of  $\text{Ca}^{++}$  into outer segments intracellularly.

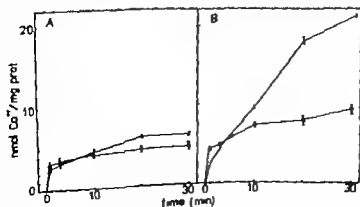


Fig. 3 Effect of ATP on accumulation of radioactive calcium into retinal outer segments. Outer segments containing about 1 mg of protein were incubated in a physiological medium containing  $1 \mu\text{Ci } ^{45}\text{CaCl}_2$  in a total volume of 450  $\mu\text{L}$ . In (B) 1 mM ATP was included (—○—) intact outer segments, (---○---) lysed outer segments. Means  $\pm$  S.E. of 4.

TABLE VI. Effect of modifications on the binding of calcium to outer segments. Phospholipid extraction was performed by suspending outer segments on ice in 90% acetone containing 10% water and 0.01% NH<sub>4</sub> for 10 min. The samples were centrifuged at 40 000 g for 10 min and washed twice with 0.1 M Tris, pH 7.4. Means of 8 determinations.

treatment	Ca <sup>++</sup> binding/μg protein % of control	
	At 5 × 10 <sup>-6</sup> M CaCl <sub>2</sub>	At 10 <sup>-4</sup> M CaCl <sub>2</sub>
control	100	100
acetone, 100 μg/ml	41	35
phospholipid extraction	41	39

-75), then phospholipids appear to be responsible for about 60% of Ca<sup>++</sup> binding of the high and the low affinity sites. Interpretation of the effect of pronase treatment is more ambiguous since the data is calculated per unit protein and pronase reduces the amount of protein. It appears, however, that protein could be responsible for the remaining Ca<sup>++</sup> binding capacity 40% of the total. Neuraminidase treatment was also applied but this failed to reduce the Ca<sup>++</sup> binding properties of outer segments.

Phospholipids were quantitated in detail as they were shown to be the major Ca<sup>++</sup> binding component of outer segments. Phospholipid/protein ratio of outer segments was 0.58 (Table VII) and the major phospholipid class was phosphatidyl ethanolamine constituting 47% of all phospholipids.

### Discussion

Isolated photoreceptor membranes have been shown to take up calcium, which process is coupled to ATP (Bownds *et al.* 1971, Newfield *et al.* 1972). Accumulation of calcium by photoreceptors has since been investigated by a number of workers (Yoshikami and Hagins 1973, Mason *et al.* 1974, Hemminki 1975) but, it has remained unestablished, whether the processes investigated have been due to binding or transport of calcium.

In this study highly pure preparations of bovine outer segments, intact and lysed, were used to differentiate between binding and transport of calcium. The intact and lysed preparations accumulated equal amounts of calcium in the absence of ATP (Fig. 3 A). This is a strong argument against the possible contribution of mitochondria to the Ca<sup>++</sup> accumu-

TABLE VII. Lipid composition of outer segments. Means of 2 to 4 determinations.

cholesterol/protein (w/w)	0.044
phospholipid/protein (w/w)	0.58
phosphatidyl ethanolamine (%)	42.7
phosphatidyl choline	36.6
phosphatidyl serine	15.3
phosphatidyl inositol	1.3
sphingomyelin	1.2
phosphoric acid	0.8
trypsinophosphatidyl choline	1.8
lysophosphatidyl ethanolamine	0.3

TABLE IV Effect of light on the binding of calcium to outer segments. Aliquots of outer segments were illuminated in room light for 5 min and incubated with unbleached controls in the dark for 15 min as described in Fig. 3. Means  $\pm$  S.E. of 4 determinations. Significant at  $p < 0.05$ .

	Calcium binding, nmol $\text{Ca}^{++}$ /mgprotein		
	Dark (A)	Light (B)	A/B 100
Incubation without A 23187			
Intact outer segments	$5.23 \pm 0.28$	$4.34 \pm 0.19$	120
Lysed outer segments	$4.72 \pm 0.08$	$3.63 \pm 0.24$	130
Incubation with A 23187			
Intact outer segments	$4.13 \pm 0.11$	$2.89 \pm 0.21$	141
Lysed outer segments	$4.03 \pm 0.30$	$2.84 \pm 0.12$	141

lation was observed (not shown). These results suggest that binding rather than transport of  $\text{Ca}^{++}$  is responsive to illumination in bovine outer segments.

In another series of experiments outer segments were first loaded with radioactive  $\text{Ca}^{++}$  by incubating them with  $^{45}\text{CaCl}_2$  in the dark. Aliquots of outer segments were then exposed to room light and the radioactivity retained was determined (Table V). No change was observed in the amount of  $\text{Ca}^{++}$  in intact outer segments. By contrast, unbleached preparations of lysed outer segments bound more  $\text{Ca}^{++}$  than the illuminated samples. This may suggest that intact plasma membranes serve as a barrier to  $\text{Ca}^{++}$  released from discs.

#### Characterization of calcium binding components

It is possible to analyse which components of the disc membrane are responsible for  $\text{Ca}^{++}$  binding by a selective removal of substances with known  $\text{Ca}^{++}$  binding properties (Hemminki 1974). The assays were performed at two  $\text{Ca}^{++}$  concentrations to probe the high and the low affinity binding sites of outer segments (Hemminki 1975). Two membrane modifications tested produced marked decrease in  $\text{Ca}^{++}$  binding to outer segments: digestion with pronase and extraction of phospholipids (Table VI). Each of the two reduced  $\text{Ca}^{++}$  binding by about 60%. If it is assumed that extraction of phospholipids is complete (see Hemminki 1975),

TABLE V Effect of light on the retention of calcium by outer segments preloaded with  $^{45}\text{CaCl}_2$ . Preparation of outer segments (1 mg protein in 400  $\mu\text{l}$  of 0.45 M sucrose) was incubated with  $^{45}\text{CaCl}_2$  (1.37  $\mu\text{Ci}$ ) in the dark for 15 min. 15 ml of 0.25 M sucrose were added and the sample centrifuged at 20 000 g for 15 min. The pellet was suspended in 1.6 ml of 0.45 M sucrose divided in two. One half was maintained in the dark and the other half was illuminated in room light for 5 min. Aliquots of 250  $\mu\text{l}$  were collected on Millipore filters and analysed as described in Materials and Methods. Means  $\pm$  S.E. of 6 determinations. Significant at  $p < 0.05$  by paired Student's *t*-test.

	Calcium retained nmol $\text{Ca}^{++}$ /mg protein		A/B 100
	Dark (A)	Light (B)	
Intact outer segments	$0.835 \pm 0.026$	$0.848 \pm 0.049$	99
Lysed outer segments	$0.613 \pm 0.04$	$0.513 \pm 0.030$	120

VI. Effect of modifications on the binding of calcium to outer segments. Phospholipid extraction was performed by suspending outer segments on ice in 90% acetone containing 10% water and 0.01 M NH<sub>4</sub> for 10 min. The samples were centrifuged at 40 000 g for 10 min and washed twice with 0.1 M Tris, pH 7.4. Means of 8 determinations.

treatment	Ca <sup>++</sup> binding/ing protein % of control	
	At 5 × 10 <sup>-6</sup> M CaCl <sub>2</sub>	At 10 <sup>-6</sup> M CaCl <sub>2</sub>
intact	100	100
lys, 100 µg/ml	41	35
phospholipid extraction	41	39

—5), then phospholipids appear to be responsible for about 60% of Ca<sup>++</sup> binding of the II and the low affinity sites. Interpretation of the effect of pronase treatment is more ambiguous since the data is calculated per unit protein and pronase reduces the amount of protein. It appears, however, that protein could be responsible for the remaining Ca<sup>++</sup> binding capacity 40% of the total. Neuraminidase treatment was also applied but this seemed to reduce the Ca<sup>++</sup> binding properties of outer segments.

Phospholipids were quantitated in detail as they were shown to be the major Ca<sup>++</sup> binding component of outer segments. Phospholipid/protein ratio of outer segments was 0.58 (Table VII) and the major phospholipid class was phosphatidyl ethanolamine constituting 43.7% of all phospholipids.

### Discussion

Isolated photoreceptor membranes have been shown to take up calcium, which process is coupled by ATP (Bownds *et al.* 1971, Neufeld *et al.* 1972). Accumulation of calcium by photoreceptors has since been investigated by a number of workers (Yoshikami and Hagins 1973, Mason *et al.* 1974, Hemminki 1975) but, it has remained unestablished, whether the processes investigated have been due to binding or transport of calcium.

In this study highly pure preparations of bovine outer segments, intact and lysed, were used to differentiate between binding and transport of calcium. The intact and lysed preparations accumulated equal amounts of calcium in the absence of ATP (Fig. 3 A). This is a strong argument against the possible contribution of mitochondria to the Ca<sup>++</sup> accumula-

TABLE VII. Lysed composition of outer segments. Means of 2 to 4 determinations.

total protein (w/w)	0.044
phospholipid, protein (w/w)	0.58
phosphatidyl ethanolamine (%)	43.7
phosphatidyl choline	34.6
phosphatidyl serine	15.3
phosphatidyl inositol	1.9
phosphoglyceride	1.2
phosphoric acid	0.8
triphosphatidyl choline	1.6
triphosphatidyl ethanolamine	0.3

tion observed. Only a small net accumulation was observed after 1 min indicating that calcium may readily be taken up even by intact outer segments. ATP did not influence the initial accumulation of calcium but showed stimulation at later time points (Fig. 3B). The extent of stimulation was small in lysed outer segments, but marked in intact outer segments. These data suggested that the initial phase of  $\text{Ca}^{++}$  accumulation could be largely attributed to binding of  $\text{Ca}^{++}$  while the delayed phase, induced by ATP, reflected active transport. A calcium ionophore, A 23187 was used to confirm these observations.

The present data support those of Bownds *et al* (1971) that the ATP-stimulated accumulation of  $\text{Ca}^{++}$  may be due to a membrane transport mechanism. Although plasma membranes are generally impermeable to ATP it was shown in the present study that in outer segments may be loaded to some degree with added ATP (Table III). It is thus likely that the membrane ATP site of outer segments is intracellular in analogy to other cell types. Yet we have so far been unable to demonstrate directly entrance of radioactive ATP to outer segments (Hemminki, unpublished) which may indicate that the effective concentrations of ATP are low in outer segments.

Binding but not transport of  $\text{Ca}^{++}$  to outer segments appeared to be regulated by light (Table IV). 20 to 40% more  $\text{Ca}^{++}$  was bound in the dark. Light dependence of  $\text{Ca}^{++}$  accumulation has also been reported elsewhere. Mason *et al* (1974) described, using sonic vesicles of outer segments, that higher amounts of  $\text{Ca}^{++}$  were transported into outer segments in light than in the dark. In the present study no light dependent  $\text{Ca}^{++}$  transport was observed but the difference of the preparations must be recognized.

Differential binding of  $\text{Ca}^{++}$  to bleached and unbleached outer segments was also demonstrated by a larger retention of  $\text{Ca}^{++}$  by unbleached preparations of lysed outer segments (Table V). It may be very important that in intact outer segments no such light dependence was observed presumably due to trapping of  $\text{Ca}^{++}$  released by plasma membrane. This is in excellent agreement of the model of visual excitation proposed by Hagood (1972). This implies release of  $\text{Ca}^{++}$  from discs on absorption of photons and blockade of  $\text{Na}^{+}$  permeability at plasma membrane by  $\text{Ca}^{++}$ .

The excellent technical assistance of Mrs. Kirsti Salmela is appreciated. Electron microscopy was performed at the Electron Microscope Laboratory Helsinki. The study was supported by the Sigrid Jusélius Foundation and the National Research Council for Medical Sciences, Finland.

## References

- BOWND, D. A., GORDON WALKER, A.-C., GAIDE HUGUENIN and W. ROBINSON. Characterization and analysis of frog photoreceptor membranes. *J. gen. Physiol.* 1971. 58: 225-237.
- CARTWELL, A. H. and B. C. FRIEDMAN. Kinetics of transport of divalent cations across sarcoplasmic reticulum vesicles induced by ionophores. *Biochem. biophys. Res. Commun.* 1972. 49: 297-299.
- DAEMEN, F. J. Vertebrate rod outer segment membranes. *Biochim. biophys. Acta (Amst.)* 1973. 300: 1-233.
- FORREMAN, J. C., J. L. MONGER and B. H. COMPTON. Calcium ionophores and movement of calcium following the physiological stimulus to a secretory process. *Nature (Lond.)* 1973. 245: 249-251.
- HAGOOD, W. A. The visual process: excitatory mechanisms in the primary receptor cells. *Ann. Rev. Physiol.* 1972. 34: 131-158.
- HAGOOD, W. A., J. T. J. OSTWALD and D. BOX. The osmotic behavior of rod photoreceptor outer segment

1. JAVORSKI, K. Composition and synthesis of plasma membranes and smooth endoplasmic reticulum in dead cells of newborn rat brain. *Biochim. Biophys. Acta* (Amst.) 1973. 298. 810-816.
2. JAVORSKI, K. Calcium binding to brain plasma membranes. *Biochim. Biophys. Acta* (Amst.) 1974. 363. 113-120.
3. JAVORSKI, K. Light-induced decrease in calcium binding to isolated bovine photoreceptors. *Vision Res.* 1975. 15. 65-72.
4. JAVORSKI, K. and O. BOUVARDON. Preparation of plasma membranes from isolated cells of newborn rat brain. *Biochim. Biophys. Acta* (Amst.) 1973. 298. 75-83.
5. KIM, W. T. R. S. FAGER and P. W. ABRAHAMSON. Ion fluxes in disk membranes of retinal rod outer segments. *Nature* (Lond.) 1974. 247. 562-563.
6. LINDH, A. H., W. H. MILLER and M. W. BRIDGEMAN. Calcium binding to retinal rod disk membranes. *Biochim. Biophys. Acta* (Amst.) 1972. 266. 57-71.
7. MORGAN, S. S. STERN and T. H. MCGOWAN. A rapid, accurate method for the determination of total calcium in serum. *Anal. Chem.* 1953. 25. 813-814.
8. NATH, D. D. K. BOWEN and B. J. BALDREY. The preservation of cellular structure and enzymatic activity by aldehyde fixation. *J. cell Biol.* 1963. 17. 18-38.
9. OKADA, T. Electrical activity of vertebrate photoreceptors. *Quart. Rev. Biophys.* 1970. 3. 179-222.
10. PETERMANN, E. and W. A. HAGERM. Control of dark current in vertebrate rods and cones. In *Biochemistry and Physiology of Visual Pigments* (Ed. H. Langer), Springer, Berlin, Heidelberg and New York. 1973. 43-251.
11. RAY, M. and S. FOTTERMAN. Extraction, regeneration after bleaching, and ion-exchange chromatography of rhodopsin in Tween 80. *Arch. Biochem. Biophys.* 1973. 137. 91-99.



## Reflexogenic Contraction of the Ileo-Cecal Sphincter in the Cat Following Small or Large Intestinal Distension

By

P. E. PAHLIN and J. KEWENTER

Received 20 March, 1975

---

### Abstract

PAHLIN P. E. and J. KEWENTER. Reflexogenic contraction of the ileo-cecal sphincter in cat following small or large intestinal distension. Acta physiol. scand. 1975. 95. 126.

The effect of distension of a small or large intestinal loop on the ileo-cecal sphincter (ICS) is studied with a flow recording technique. Distension of a small or large intestinal loop, isolated from the adjacent intestine, but with its mesenteric vasculature and nervous supply intact, elicited contraction of the ICS concomitant with an inhibition of the adjacent large and small intestine. Vagal and pelvic nerve section did not affect the response to distension of the ICS nor exclusion of the adrenal glands from the circulation. The sphincter reflex could be entirely or almost entirely abolished by cutting both the splanchnic and lumbar colonic nerves, but not one or the other. Spinal anaesthesia blocked the reflex response indicating a spinal reflex arc. The reflex persisted after atropine and propranolol, while both guanethidine and phenoxybenzamine completely abolished the reflex contraction of the sphincter.

The present results indicate that the excitatory intestino-ileo-cecal sphincter reflex is a spinal reflex with the main afferent and/or efferent fibres located within the major splanchnic and lumbar colonic nerves. The excitatory motor response in the sphincter is adrenergic and mediated via  $\alpha$ -receptors.

---

Distension of a segment of jejunum or ileum, isolated from the adjacent intestine but with its vascular and nervous supply intact, elicits an inhibition of the small intestine via the so-called intestino-intestinal inhibitory reflex, which is a spinal reflex under supraspinal control (Johansson and Langston 1964, Johansson *et al.* 1965 and 1968). Similar distension of a segment of the small intestine induces an inhibition of the motility of the proximal part of the so-called intestino-colonic inhibitory reflex (Hultén 1969). In contrast, a contraction was obtained in the ileo-cecal sphincter (ICS) upon distension of a loop of the small or large intestine (Hirschman and Ivy 1931, Chang and Hsu 1942). Chang *et al.* (1942) found that splanchnic, but not vagal, section abolished the latter reflex. Hirschman and Ivy (1931) on the other hand could only eliminate the reflex by cutting both the splanchnic and lumbar colonic nerves, not one or the other. They were unable to determine the relative importance of an extrinsic or an intrinsic intramural nervous mechanism in the

tol of the sphincter. The excitatory response of the sphincter to intestinal distension  
 be, theoretically be either cholinergic, adrenergic or both as it has been shown that both  
 ergic and cholinergic nerve stimulation elicits a contraction of the ICS (Pahlm and  
 wenter 1975). Neither can a non-adrenergic, non-cholinergic mechanism be excluded as  
 as been shown that such a transmitter mechanism must be involved in the extrinsic  
 ous control of the gastrointestinal motor activity (e.g. Martinson 1965, Hultén 1969).  
 view of these partly conflicting results, it was thought of interest to investigate further  
 reflexogenic effect of intestinal distension on the ICS with respect to the nervous path-  
 ns and peripheral transmitter mechanism.

# Methods

adult cats of both sexes, deprived of food for 24 h but with free access to water were used. After iso-  
 tion with ether the anaesthesia was continued with chloralose (30 mg/kg b w i. v.). A tracheal cannula  
 inserted. The blood pressure was measured via femoral artery.

## Recording of intrasphincteric flow

The abdomen was opened by midline incision. The ileum was divided about one cm proximally to the  
 and crossing ligature was tied around the colonic wall 1-2 cm distally to the ICS. In this way the  
 -ical region was isolated from the adjacent parts of the gut but its mesenteric nervous and vascular  
 ly intact. A tube was inserted in the abdominal end of the divided ileum and fixed with ligature imme-  
 diately above the ICS. The ileal motor activity could thereby not influence the flow through the sphincter.  
 rectological saline (38°C) was led from reservoir to the sphincter via the tube. Hence the cross-sectional  
 of the reservoir was large (70 cm<sup>2</sup>) the perfusion pressure could be kept almost constant by means of  
 regulation of the saline. The inflow pressure was usually set at about 5 cm H<sub>2</sub>O. The saline reaching the  
 rectal colon through the sphincter was drained by funnel inserted with its wider end through an  
 incision in the antimesenteric border of the caecum. The saline was led to Gaddum-recorder (Gaddum  
 1939) and volume changes in the recorder reflected the flow through the sphincter. The changes were  
 measured via penon-recorder on smoked drum.

## Recording of intestinal motility. Distension of intestinal loop

Intestinal loops from the jejunum, ileum (about 15 cm in length) and colon (10 cm) were prepared either  
 to record the motor activity or to be used for intestinal distension. The small intestinal loops were isolated  
 in the adjacent gut by transection of the wall and the colonic loops by crushing ligatures. The mesenteric  
 vessels and blood supply to the loops was intact. The proximal ends of the small intestinal loops were  
 tied by ligatures and glass cannulae was inserted into the distal end and fixed with ligature. In the  
 ileal loop the cannulae as inserted through an incision in the antimesenteric border and fixed with  
 suture string suture. The loops used for recording intestinal motility were connected to adaptable reservoirs  
 adapted to volume recorder. The systems were filled with normal saline at body temperature. — The loops  
 used for distension were coupled to reservoirs that could easily be adapted to different heights. These  
 reservoirs were also filled with normal saline at body temperature. Distension pressures from 30-100 cm  
 H<sub>2</sub>O were used. The interval between the distensions was usually 10 min or more.

## Anal anaesthesia. Nervous stimulation

5 cats spinal anaesthesia was used to study whether the ileosteo-ileo-caecal reflex is spinal reflex or  
 mediated via preganglionic reflex arc. Through cervical laminectomy at C VI and C VII this poly-  
 chylene catheter was inserted retrogradually about 2 cm in the cranial direction. 2 ml of the anaesthetic  
 was injected through the catheter. Artificial respiration had to be maintained by means of respiratory  
 pump in 3 of the animals. The left major splanchnic nerve was divided in 4 of the cats subjected to spinal  
 anaesthesia. A silver coil electrode was attached on the distal end of the cat splanchnic nerve for stimulation.  
 The square wave impulses by means of Grass stimulator (S4E) at supramaximal voltages and duration  
 1-12 V 3-5 ms. The length of the stimulus train was 30-60

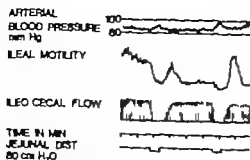


Fig. 1 Effect of jejunal distension on the arterial pressure, ileal motility and transsphincteric flow. Jejunum distended elicited a slight increase of the blood pressure. Inhibition of the ileal motility and a contraction of sphincter. Adrenal glands ligated.

Intestinal distension was performed before and after cervical vagotomy in 9 cats. In 2 of these the vagal nerves were cut as well.

The 2 major splanchnic nerves were divided subdiaphragmatically in 9 animals. In 6 of these the effect of intestinal distension on the transsphincteric flow was also studied after the lumbar colonic nerves had been cut as well. In 3 cats the effect of intestinal distension was studied when only the lumbar colonic nerves had been cut.

Catecholamines from the adrenal glands are known to be a potent factor in the regulation of the ileal motor activity (Kock 1959). In order to exclude the hormonal influence from the adrenals, the two of these glands were tied off. In 2 cats the ligature was performed during the experiment.

#### Drugs

The following drugs were administered intravenously: To block the "muscarinic" cholinergic receptors: atropine (Atropine sulphate, Merck) 1 mg/kg b.w.

To block the adrenergic  $\alpha$  receptors: phenoxybenzamine (Dibenzylamine<sup>®</sup> Smith, Kline & French) 3 mg/kg b.w.

To block the adrenergic  $\beta$ -receptors: propranolol (Inderal<sup>®</sup> ICI) 1-2 mg/kg b.w.

To block the adrenergic transmission: guanethidine (Ismelin<sup>®</sup> Ciba, Basel) 3 mg/kg b.w.

To induce spinal anesthesia: Tetracaine (Tetracain<sup>®</sup> Apoteksbolaget, Sweden) 10 mg/ml, or mepivacaine (Carbocain<sup>®</sup> Nobel Pharma) 20 mg/ml.

#### Results

Distension of a jejunal (22 cats), ileal (6 cats) or proximal colonic loop (4 cats) reduced or stopped the transsphincteric flow in all cats. The sphincter contraction was obtained at a distension pressure between 30 and 100 cm H<sub>2</sub>O. Concomitant with the sphincter contraction there was an inhibition of tone and motor activity of the isolated large or small intestinal loops (Fig. 1). The results of the following experiments were all obtained by distension of a small intestinal loop.

In order to investigate whether the tone of the ICS prior to distension influenced the sphincteric response, the intestinal distension was repeated after the tone of the sphincter had been increased either by efferent splanchnic nerve stimulation (2 cats) or by distension of an other intestinal segment (1 cat). No relaxation of the sphincter could be recorded during these conditions.

No difference was observed in the sphincteric response to intestinal distension before and after the adrenal glands had been tied off from the circulation by ligatures (2 animals).

The intestino-ileo-cecal sphincteric reflex was unchanged after the vagal nerves had been cut in the neck and also when both the vagal and pelvic nerves were cut.

The reflexogenic sphincter contraction was unchanged or only reduced to a minor extent

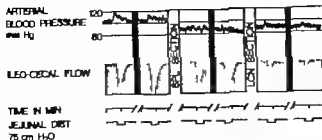


Fig. 2. Effect of splanchnic and lumbar colonic nerve (LCN) section on the sphincter contraction elicited by jejunal distension. Note that splanchnic section did not affect the reflex contraction of ICS. When the lumbar colonic nerves were cut as well the ICS response to jejunal distension was almost completely abolished. Adrenal glands ligated.

after bilateral splanchnic nerve section. However when the lumbar colonic nerves were cut as well, the contraction was totally or almost totally abolished (Fig. 2). When the lumbar colonic nerves, on the other hand, was cut first, the reflex response could still be elicited (Fig. 3).

In order to investigate whether the Intestine-Ileo-cecal sphincteric reflex was mediated in the abdominal ganglia or was dependent upon nervous connection with the spinal cord, experiments were performed. The ganglionic sympathetic blockade was induced by means of spinal anesthesia. The results obtained in one of these experiments are illustrated in Fig. 4. At in the panel A, distension of an ileal loop elicited contraction of the ICS. Splanchnic nerve stimulation (*b*) at 8 Hz, 6 V and 4 ms induced a contraction of the sphincter and an increase in blood pressure. Spinal anesthesia was then performed (between panel A and B in the figure). Ileal distension (*a* in panel B) no longer causes any contraction of the ICS. The effect of splanchnic nerve stimulation (*b'* in panel B) remained unchanged, however. These results indicate that the reflex is spinal. Contraction of the ICS to intestinal distension (*a* in panel C) was again recorded after the spinal anesthesia had worn off. In 3 of the 5 animals spinal anesthesia blocked the reflexogenic contraction of the sphincter. In the remaining 2 animals the spinal anesthesia strongly reduced the reflex contraction but the response was not totally abolished, probably due to incomplete spinal anesthesia.

Atropine did not block the reflex contraction of the sphincter (6 cats), while guanethidine (3 cats) completely abolished the reflex. Propranolol (7 cats) did not block the reflex while it was completely eliminated by phenoxybenzamine (2 cats).

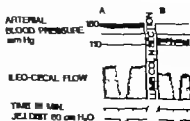


Fig. 3. Effect of jejunal distension on the transsphincter flow before and after section of the lumbar colonic nerves. Splanchnic nerves intact. Vagal and pelvic nerves cut. Both adrenal glands ligated.

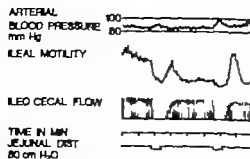


Fig. 1 Effect of jejunal distension on the arterial blood pressure, ileal motility and transsphincteric flow. Jejunal distension elicited a slight increase of the blood pressure, inhibition of the ileal motility and a contraction of sphincter. Adrenal glands ligated.

Intestinal distension was performed before and after cervical vagotomy in 9 cats. In 2 of these the pelvic nerves were cut as well.

The 2 major splanchnic nerves were divided subdiaphragmatically in 9 animals. In 6 of these animals effect of intestinal distension on the transsphincteric flow was also studied after the lumbar colic nerves had been cut as well. In 3 cats the effect of intestinal distension was studied when only the lumbar colic nerves had been cut.

Catecholamines from the adrenal glands are known to be a potent factor in the regulation of the ileal motor activity (Kock 1959). In order to exclude the hormonal influence from the adrenals, the veins of these glands were tied off. In 2 cats the ligature was performed during the experiment.

#### Drugs

The following drugs were administered intravenously. To block the "muscarinic" cholinergic receptors: atropine (Atropine sulphate, Merck) 1 mg/kg b.w.

To block the adrenergic  $\alpha$ -receptors: phenoxybenzamine (Dibenzylin® Smith, Kline & French) 5 mg b.w.

To block the adrenergic  $\beta$ -receptors: propranolol (Inderal® ICI) 1–2 mg/kg b.w.

To block the adrenergic transmission: guanethidine (Isometin® Ciba, Basel) 3 mg/kg b.w.

To induce spinal anesthesia: Tetracain grave® (Apoteksbolaget Sweden) 10 mg/ml, or mepivacaine chlo (Carbocain® Nobel Pharma) 20 mg/ml.

## Results

Distension of a jejunal (22 cats), ileal (6 cats) or proximal colonic loop (4 cats) reduced or stopped the transsphincteric flow in all cats. The sphincter contraction was obtained at distension pressure between 30 and 100 cm H<sub>2</sub>O. Concomitant with the sphincter contraction there was an inhibition of tone and motor activity of the isolated large or small intestinal loops (Fig. 1). The results of the following experiments were all obtained by distension of a small intestinal loop.

In order to investigate whether the tone of the ICS prior to distension influenced the sphincteric response, the intestinal distension was repeated after the tone of the sphincter had been increased either by efferent splanchnic nerve stimulation (2 cats) or by distension of an other intestinal segment (1 cat). No relaxation of the sphincter could be recorded during these conditions.

No difference was observed in the sphincteric response to intestinal distension before and after the adrenal glands had been tied off from the circulation by ligatures (2 animals).

The intestino-ileo-cecal sphincteric reflex was unchanged after the vagal nerves had been cut in the neck and also when both the vagal and pelvic nerves were cut.

The reflexogenic sphincter contraction was unchanged or only reduced to a minor extent

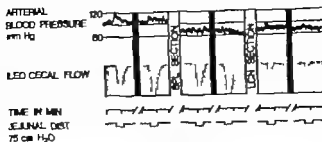
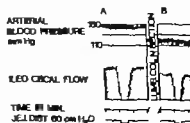


Fig. 1. Effect of splanchnic and lumbar colonic nerve (LCN) section on the sphincter contraction elicited by jejunal distension. Note that splanchnic section did not affect the reflex contraction of ICS. When the lumbar colonic nerves were cut as well the ICS response to jejunal distension was almost completely unaltered. Adrenal glands ligated.

After bilateral splanchnic nerve section. However when the lumbar colonic nerves were cut as well, the contraction was totally or almost totally abolished (Fig. 2). When the lumbar colonic nerves, on the other hand, was cut first, the reflex response could still be elicited (Fig. 3).

In order to investigate whether the intestino-ileo-cecal sphincteric reflex was mediated by the abdominal ganglia or was dependent upon nervous connection with the spinal cord, experiments were performed. The ganglionic sympathetic blockade was induced by means of spinal anesthesia. The results obtained in one of these experiments are illustrated in Fig. 4. In the panel A, distension of an ileal loop elicited a contraction of the ICS. Splanchnic nerve stimulation (b) at 8 Hz 6 V and 4 ms induced a contraction of the sphincter and an increase in blood pressure. Spinal anesthesia was then performed (between panel A and B in the figure). Ileal distension (c) in panel B no longer causes any contraction of the ICS. The effect of splanchnic nerve stimulation (b) in panel B remained unchanged, however these results indicate that the reflex is spinal. Contraction of the ICS to intestinal distension in panel C was again recorded after the spinal anesthesia had worn off. In 3 of the 5 animals spinal anesthesia blocked the reflexogenic contraction of the sphincter. In the remaining 2 animals the spinal anesthesia strongly reduced the reflex contraction but the response was not totally abolished, probably due to incomplete spinal anesthesia. Atropine did not block the reflex contraction of the sphincter (6 cats), while guanethidine (3 cats) completely abolished the reflex. Propranolol (7 cats) did not block the reflex while it was completely eliminated by phenoxybenzamine (2 cats).

Fig. 2. Effect of jejunal distension on the transsphincter flow after and after section of the lumbar colonic nerves. Splanchnic nerves intact. Vagal and pelvic nerves cut. Both adrenal glands ligated.



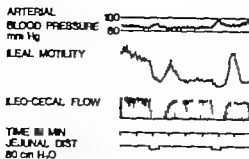


Fig. 1 Effect of jejunal distension on the arterial blood pressure, ileal motility and transsphincteric flow. Jejunum distension elicited a slight increase in the blood pressure, inhibition of the ileal motility and a contraction of the sphincter. Adrenal glands ligated.

Intestinal distension was performed before and after cervical vagotomy in 9 cats. In 2 of these the pelvic nerves were cut as well.

The 2 major splanchnic nerves were divided subdiaphragmatically in 9 animals. In 6 of these animals the effect of intestinal distension on the transsphincteric flow was also studied after the lumbar colic nerves had been cut as well. In 3 cats the effect of intestinal distension was studied when only the lumbar colic nerves had been cut.

Catecholamines from the adrenal glands are known to be a potent factor in the regulation of the intestinal motor activity (Kock 1959). In order to exclude the hormonal influence from the adrenals, the veins of these glands were tied off. In 2 cats the ligature was performed during the experiment.

#### Drugs

The following drugs were administered intravenously. To block the "muscarinic" cholinergic receptors atropine (Atropine sulphate, Merck) 1 mg/kg b.w.

To block the adrenergic  $\alpha$ -receptors, phenotyzbenzamine (Dibucaine® Smith, Kline & French) 5 mg/kg b.w.

To block the adrenergic  $\beta$ -receptors propranolol (Inderal® ICI) 1-2 mg/kg b.w.

To block the adrenergic transmission guanethidine (Ismelin® Ciba Basel) 3 mg/kg b.w.

To induce spinal anesthesia Tetracain grise® (Apoteksbolaget, Sweden) 10 mg/ml, or meprocaine (Carbocain® Nobel Pharma) 20 mg/ml.

#### Results

Distension of a jejunal (22 cats), ileal (6 cats) or proximal colonic loop (4 cats) reduced or stopped the transsphincteric flow in all cats. The sphincter contraction was obtained at distension pressure between 30 and 100 cm H<sub>2</sub>O. Concomitant with the sphincter contraction there was an inhibition of tone and motor activity of the isolated large or small intestinal loops (Fig. 1). The results of the following experiments were all obtained by distension of a small intestinal loop.

In order to investigate whether the tone of the ICS prior to distension influenced the sphincteric response, the intestinal distension was repeated after the tone of the sphincter had been increased either by efferent splanchnic nerve stimulation (2 cats) or by distension of an other intestinal segment (1 cat). No relaxation of the sphincter could be recorded during these conditions.

No difference was observed in the sphincteric response to intestinal distension before and after the adrenal glands had been tied off from the circulation by ligatures (2 animals).

The intestino-ileo-cecal sphincteric reflex was unchanged after the vagal nerves had been cut in the neck and also when both the vagal and pelvic nerves were cut.

The reflexogenic sphincter contraction was unchanged or only reduced to a minor extent

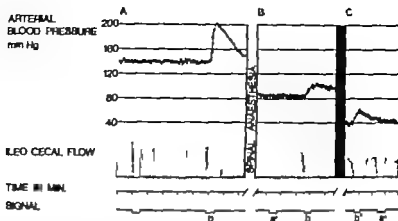


Fig. 4. Effect of spinal anesthesia on the ICS response to ileal distension and splanchnic nerve stimulation. a, a and a ileal distension. b, b and b splanchnic nerve stimulation (8 Hz, 6 V and 4 ms). Note that ICS response to ileal distension disappears after spinal anesthesia (Panel B). This response could be elicited again after spinal anesthesia had worn off (Panel C). The effect of splanchnic nerve stimulation remains unchanged throughout the experiment. Both adrenal glands were ligated. The vagal nerves were cut in the neck.

### Discussion

The present experiments have shown that distension of a small or large intestinal loop elicits a contraction of the ICS concomitant with an intestinal inhibition. This effect is mediated *via* an extrinsic mechanism as the distended intestinal loop as well as the K region was isolated from the adjacent gut, but with the mesenteric vascular and nerve supply intact. A humoral adrenergic mechanism *via* adrenaline released from the adrenal glands, could be excluded as the cause of the reflex contraction of the ICS as the reflex remained unchanged after the exclusion of the adrenal glands from the circulation.

Cervical vagotomy did not influence the intestino-ileo-cecal sphincteric reflex, which is in accordance with the results of Chang and Hau (1942). The reflex activation of the sphincter could also be elicited in cats in which both the pelvic and vagal nerves had been cut. As the reflex contraction could also be elicited after atropine it is concluded that the intestino-ileo-cecal excitatory reflex is non-cholinergic and not mediated *via* the cranial or sacral parasympathetic nervous system.

Guanethidine blocked the reflex, which makes an adrenergic peripheral transmitter mechanism likely. The reflex contraction of the sphincter was also blocked by phenoxybenzamine but not by propranolol. It is therefore concluded that the sphincteric contraction elicited by distension of an intestinal loop is mediated *via* an  $\alpha$  adrenergic receptor mechanism. This is in accordance with an investigation of the adrenergic receptor mechanism in the ICS which showed that contraction of the sphincter elicited by adrenergic nerve stimulation was mediated *via*  $\alpha$ -adrenergic receptors (Pahlin and Kewenter to be published).

The disappearance of the intestino-ileo-cecal sphincteric reflex after spinal anesthesia indicates that the reflex response of the ICS to intestinal distension is dependent upon nervous connection with the spinal cord and cannot be elicited *via* decentralized abdominal ganglia.

The reflex could be activated even after the major splanchnic nerves had been cut, although



Reflex contraction of the ICS was decreased in some of the experiments. When the lumbar colonic nerves were cut as well, the reflex was totally or almost totally abolished. The effect of lumbar colonic nerve section on the sphincter reflex may suggest that the whole or almost the whole reflex is mediated via the lumbar colonic nerves. However, when the lumbar colonic nerves were divided while the splanchnic nerves were intact the response was unchanged or only blocked to a minor extent. This suggests that both the splanchnic and the lumbar colonic nerves are involved in the mediation of the reflex contraction of the ICS during distension of a small intestinal loop. In some of the experiments a small reflex contraction of the ICS persisted even though the major splanchnic and lumbar colonic nerves were cut. This must indicate that the reflex can be at least partly mediated via nervous connections with the spinal cord outside the main sympathetic nerve trunks, i.e. the major splanchnic and lumbar colonic nerves.

Chang and Hsu (1942) found that the reflexogenic response of the sphincter varied with the sphincter tone. Thus, distension induced an inhibition of the sphincter when the tone was high and vice versa. In the present experiments intestinal distension did not induce relaxation of the sphincter when the tone in the ICS had been increased by splanchnic nerve stimulation or distension of another intestinal loop. Instead, a further contraction of the sphincter was obtained. Thus, no inhibitory fibres to the sphincter could be demonstrated in the present study. However, this does not exclude the existence of such fibres, which might possibly be activated under other experimental conditions.

The intestino-ileo-cecal reflex is with respect to the reflex limbs and nervous connection in the spinal cord very similar to the intestino-intestinal inhibitory reflex (Johansson and Larsson 1964) and the results obtained by Hultén (1969) for the intestino-colonic inhibitory reflex. In fact, it seems reasonable to assume that these reflexes, which are elicited by intestinal distension, are induced simultaneously and mediated via similar reflex arcs. This is supported by the present experiments, in which the motor activity of the small and large intestine was recorded concordantly with the transsphincteric flow.

Distension of an intestinal loop has been shown to produce a rapid reflex inhibition of the stomach and the small and large intestines (Johansson and Langston 1964, Johansson and Larsson 1966, Hultén 1969) and of the sphincter of Oddi in dogs (Nakayama 1969). Similarly, astral distension elicits a reflex inhibition of the corpus-fundus via an adrenergic spinal reflex (Abrahamsson 1974). Contrary to these reflexes, the intestino-ileo-cecal transsphincteric reflex is an excitatory reflex. The functional significance of this excitatory reflex is unclear.

The results of the present study differ from the findings in man by Cohen *et al.* (1968) and in dogs by Kelley *et al.* (1966). These authors found that ileal balloon distension elicited a pressure fall in the sphincter while they obtained a rise in the intrasphincteric pressure with colonic balloon distension. Distension of the intestine above as well as below the ICS always elicited a contraction of the sphincter in the present experiments. Whether this difference is due to a species difference or some other factor remains to be investigated.

This work was supported by the Swedish Medical Research Council (project no B75-17X 577-11A) and Göteborgs Läkarförening.

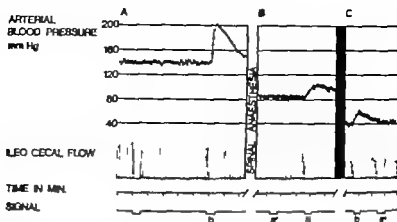


Fig. 4 Effect of spinal anesthesia on the ICS response to ileal distension and splanchnic nerve stimulation. a, a and a ileal distension. b, b and b splanchnic nerve stimulation (8 Hz, 6 V and 4 ms). Note that the ICS response to ileal distension disappears after spinal anesthesia (Panel B). This response could again appear after spinal anesthesia had worn off (Panel C). The effect of splanchnic nerve stimulation remained unchanged throughout the experiment. Both adrenal glands were ligated. The vagal nerves were cut at the neck.

### Discussion

The present experiments have shown that distension of a small or large intestinal elicits a contraction of the ICS concomitant with an intestinal inhibition. This effect is mediated *via* an extrinsic mechanism as the distended intestinal loop as well as the region was isolated from the adjacent gut, but with the mesenteric vascular and nerve supply intact. A humoral adrenergic mechanism *via* adrenaline released from the adrenal glands, could be excluded as the cause of the reflex contraction of the ICS as the response remained unchanged after the exclusion of the adrenal glands from the circulation.

Cervical vagotomy did not influence the intestino-ileo-cecal sphincteric reflex, which is in accordance with the results of Chang and Hau (1942). The reflex activation of the sphincter could also be elicited in cats in which both the pelvic and vagal nerves had been cut. A reflex contraction could also be elicited after atropine it is concluded that the intestino-cecal excitatory reflex is non-cholinergic and not mediated *via* the cranial or sacral sympathetic nervous system.

Guanethidine blocked the reflex, which makes an adrenergic peripheral transmitter mechanism likely. The reflex contraction of the sphincter was also blocked by phenoxybenzamine but not by propranolol. It is therefore concluded that the sphincteric contraction elicited by distension of an intestinal loop is mediated *via* an  $\alpha$ -adrenergic receptor mechanism. This is in accordance with an investigation of the adrenergic receptor mechanism in the ileum which showed that contraction of the sphincter elicited by adrenergic nerve stimulation was mediated *via*  $\alpha$ -adrenergic receptors (Pahlin and Kewenter to be published).

The disappearance of the intestino-ileo-cecal sphincteric reflex after spinal anesthesia indicates that the reflex response of the ICS to intestinal distension is dependent on a nervous connection with the spinal cord and cannot be elicited *via* decentralized abdominal ganglia.

The reflex could be activated even after the major splanchnic nerves had been cut, altho

reflex contraction of the ICS was decreased in some of the experiments. When the lumbar colonic nerves were cut as well, the reflex was totally or almost totally abolished. The effect of lumbar colonic nerve section on the sphincter reflex may suggest that the whole or almost the whole reflex is mediated via the lumbar colonic nerves. However when the splanchnic nerves were divided while the splanchnic nerves were intact the response was unchanged or only blocked to a minor extent. This suggests that both the splanchnic and the lumbar colonic nerves are involved in the mediation of the reflex contraction of the ICS during distension of a small intestinal loop. In some of the experiments a small reflex contraction of the ICS persisted even though the major splanchnic and lumbar colonic nerves were cut. This must indicate that the reflex can be at least partly mediated via nervous actions with the spinal cord outside the main sympathetic nerve trunks, i.e. the major splanchnic and lumbar colonic nerves.

Yang and Hsu (1942) found that the reflexogenic response of the sphincter varied with sphincter tone. Thus, distension induced an inhibition of the sphincter when the tone was high and vice versa. In the present experiments intestinal distension did not induce relaxation of the sphincter when the tone in the ICS had been increased by splanchnic nerve stimulation or distension of another intestinal loop. Instead, a further contraction of the sphincter was obtained. Thus, no inhibitory fibres to the sphincter could be demonstrated in the present study. However this does not exclude the existence of such fibres, which might possibly be activated under other experimental conditions.

The ileostoma-ileo-cecal reflex is with respect to the reflex limbs and nervous connection in the spinal cord very similar to the ileostoma-intestinal inhibitory reflex (Johansson and Upton 1964) and the results obtained by Hultén (1969) for the ileostoma-colonic inhibitory reflex. In fact, it seems reasonable to assume that these reflexes, which are elicited by intestinal distension, are induced simultaneously and mediated via similar reflex arcs. This is supported by the present experiments, in which the motor activity of the small and large intestine was recorded concomitant with the transsphincteric flow.

Distension of an intestinal loop has been shown to produce a rapid reflex inhibition of the stomach and the small and large intestines (Johansson and Langston 1964, Jansson and Eriksson 1966, Hultén 1969) and of the sphincter of Oddi in dogs (Nakayama 1969). Similarly aural distension elicits a reflex inhibition of the corpus-fundus via an adrenergic spinal reflex (Abrahamsson 1974). Contrary to these reflexes, the ileostoma-ileo-cecal haemorrhagic reflex is an excitatory reflex. The functional significance of this excitatory reflex is unclear.

The results of the present study differ from the findings in man by Cohen *et al.* (1968) and in dogs by Kelley *et al.* (1966). These authors found that ileal balloon distension elicited pressure fall in the sphincter while they obtained a rise in the intrasphincteric pressure with colonic balloon distension. Distension of the intestine above as well as below the ICS always elicited a contraction of the sphincter in the present experiments. Whether this difference is due to species differences or some other factor remains to be investigated.

## References

- ABRAHAMSON H., Reflex adrenergic inhibition of gastric motility elicited from the gastric antrum. *J. physiol. scand.* 1974, 90, 14-24.
- CHANG, P. Y. and F. Y. HSU. The localization of the intestinal inhibitory reflex arc. *Quart. J. exp. Med.* 1944, 31, 311-318.
- COHEN S., L. D. HARRIS and R. LEVITAN. Manometric characteristics of the human ileocecal junction. *Gastroenterology* 1968, 54, 77-78.
- GADDUM, J. H., An outflow recorder. *J. Physiol. (Lond.)* 1979, 67, XI-XII.
- HINRICHSSEN, J. and A. C. IVY. Studies of the ileo-cecal sphincter of the dog. *Amer. J. Physiol.* 1949, 494-507.
- HULTÉN, L., Extrinsic nervous control of colonic motility and blood flow. An experimental study cat. *Acta physiol. scand.* 1969, Suppl. 333.
- JANSSON G. and J. MARTINSON. Studies on the ganglionic site of action of sympathetic outflow to stomach. *Acta physiol. scand.* 1966, 68, 184-194.
- JOHANSSON B. and J. B. LANGSTON. Reflex influence of mesenteric afferents on renal, intestinal and blood flow and on intestinal motility. *Acta physiol. scand.* 1964, 61, 400-412.
- JOHANSSON, B., O. JOHANSSON and B. LUNG. Supraspinal control of the intestino-intestinal inhibition. *Acta physiol. scand.* 1963, 63, 442-449.
- JOHANSSON, B., O. JOHANSSON and B. LUNG. Tonic supraspinal mechanisms influencing the intestino-intestinal inhibitory reflex. *Acta physiol. scand.* 1968, 72, 200-204.
- KELLER, M. L. Jr, E. A. GORDON and J. A. DEWLESE. Pressure response of canine ileocecal junction zone to intestinal distention. *Amer. J. Physiol.* 1966, 211, 614-618.
- KOCK, N. G., An experimental analysis of mechanisms engaged in reflex inhibition of intestinal motility. *Acta physiol. scand.* 1959, Suppl. 164.
- MARTINSON, J. V. gall relaxation of the stomach. *Acta physiol. scand.* 1965, 65, Suppl. 35.
- NAKAYAMA, S., Effect of distension of the small intestine on the movements of the gall bladder and sphincter of Oddi. *Acta Med. Okazawa* 1969, 23, 21-26.
- PAHLIN P. E. and J. HEWENTER. The direct sympathetic nervous control of the cat ileo-cecal sphincter. *Amer. J. Physiol.* 1975. Submitted for publication.

## Intracellular Distribution of Amines Taken up by Rat Mast Cells In Vitro

By

ANDERS BERGENDORFF

Received 27 March 1975

### Abstract

BERGENDORFF A. Intracellular distribution of amines taken up by rat mast cell in vitro. *Acta physiol. scand.* 1975. 95. 133-141.

Mast cells isolated from rat peritoneal and pleural cavities were incubated *in vitro* with radioactively-labelled histamine (Hi), 5-hydroxytryptamine (5-HT), dopamine (DA), noradrenaline (NA), tyramine (T), phenylethylamine (PEA), tryptamine (TrpA), epinephrine (Eph) or amphetamine (Amph). All these amines were taken up by the mast cells. The dose-response curves for the compound 48/80-induced release of endogenous Hi and for the various amines taken up by the cells were compared. The release curves for 5-HT, DA, NA and TA were found to be similar to that for endogenous Hi, while those for PEA, Eph and Amph were different from that for endogenous Hi. The uptake of Hi, 5-HT, DA, PEA, NA and Eph into granules in mast cells was studied. Membrane-bound granules were obtained by sonication of mast cells incubated with the respective amine, followed by differential centrifugation. The amine content of these granules was then measured. Hi, 5-HT and DA were found to be mainly localized to the granules, while smaller proportions of the PEA, TrpA and Eph were found there, the rest being located cytoplasmically. The present results suggest that, when taken up by rat mast cells, even amines which are not endogenous to the cells are stored in the same way as the endogenous amines Hi and 5-HT.

Many authors (Purano and Green 1964, Cabot and Hagermark 1966, Jansson 1970, Frisk, Olsson and Uvnäs 1972 and Heisler and Uvnäs 1972) have shown that rat mast cells take up not only the amines which occur in them naturally—histamine (Hi) and 5-hydroxytryptamine (5-HT)—but also noradrenaline (NA) and dopamine (DA). Granules isolated from water-lysed rat mast cells have been shown to bind various amines in a rather unselective manner (Bergendorff and Uvnäs 1973). The main constituent of the granule matrix is a heparin-protein complex (Bergqvist, Samuelsson and Uvnäs 1971) which has the properties of a weak cation exchange material. The binding sites on the complex are probably carboxyl groups (Uvnäs, Åberg and Bergendorff 1970), to which the amines are linked ionically. Since granules isolated in the above way are devoid of an intact membrane, there is no barrier to ion movements between the granules and the surrounding medium.

The object of the present study was to determine if the unselective cation storage mechanism found in granules (see above) was also operative inside the mast cell. Two separate

## References

- ABRAHAMSSON, H., Reflex adrenergic inhibition of gastric motility elicited from the gastric antrum *physiol. scand.* 1974. 90 14-24.
- CHANG, F Y and F Y HSU The localization of the intestinal inhibitory reflex arc. *Quart. J. exp. Physiol.* 1942. 31 311-318.
- COHEN S. L. D. HARRIS and R. LEVITAN, Manometric characteristics of the human ileocecal junction. *Gastroenterology* 1968. 54 72-75.
- GADDUM, J. H., An outflow recorder *J. Physiol. (Lond.)* 1929. 67 XI-XII.
- HODGKINSON, J. and A. C. IVY Studies of the ileo-cecal sphincter of the dog. *Amer. J. Physiol.* 1937. 151 494-507.
- HULTÉN L., Extrinsic nervous control of colonic motility and blood flow. An experimental study in cat. *Acta physiol. scand.* 1969. Suppl. 335.
- JANSSON G. and J. MARTINSON, Studies on the ganglionic site of action of sympathetic outflow to the stomach. *Acta physiol. scand.* 1966. 68 184-192.
- JOHANSSON, B. and J. B. LANOSTON, Reflex influence of mesenteric afferents on renal, intestinal and blood flow and on intestinal motility *Acta physiol. scand.* 1964. 61 400-412.
- JOHANSSON, B., O. JOHANSSON and B. LJUNG, Supraspinal control of the ileostino-intestinal inhibitory reflex. *Acta physiol. scand.* 1965. 63. 442-449.
- JOHANSSON, B., O. JOHANSSON and B. LJUNG, Tonic supraspinal mechanisms influencing the ileostino-intestinal inhibitory reflex. *Acta physiol. scand.* 1968. 72. 200-204.
- KELLEY M. L. Jr E. A. GORDON and J. A. DEWINTER, Pressure response of canine ileocolonic junction to intestinal distention *Amer. J. Physiol.* 1966. 211 614-618.
- KOCK, N. O. An experimental analysis of mechanisms engaged: reflex inhibition of intestinal motility. *Acta physiol. scand.* 1959. 3 pp. 164.
- MARTINSON J. Vagal relaxation of the stomach. *Acta physiol. scand.* 1965. 65. Suppl. 255.
- NAKAYAMA S., Effect of distension of the small intestine on the movements of the gall bladder and sphincter of Oddi. *A to Med. Okayama* 1969. 23. 21-26.
- PAHLIN, P. E. and J. KEWENTER, The direct sympathetic nervous control of the cat ileo-cecal sphincter. *Amer. J. Physiol.* 1975. Submitted for publication.

## Intracellular Distribution of Amines Taken up by Rat Mast Cells In Vitro

By

ANDERS BERGENDORFF

Received 27 March 1975

### Abstract

BERGENDORFF A. Intracellular distribution of amines taken up by rat mast cell in vitro. *Acta physiol. scand.* 1975. 95. 133-141.

Mast cells isolated from rat peritoneal and pleural cavities were incubated in vitro with radioactively-labelled histamine (Hi), 5-hydroxytryptamine (5-HT), dopamine (DA), noradrenaline (NA), tyramine (T), phenylethylamine (PhEA), tryptamine (TrpA), epinephrine (Eph) or amphetamine (Amph). All these amines were taken up by the mast cells. The dose-response curves for the compound 48/80-induced release of endogenous Hi and for the various amines taken up by the cells were compared. The release curves for 5-HT, DA, NA and TA were found to be similar to that for endogenous Hi, while those for PhEA, T, Eph and Amph were different from that for endogenous Hi. The uptake of Hi, 5-HT, DA, PhEA, T and Eph into granules in mast cells was studied. Membrane-bound granules were obtained by isolation of mast cells incubated with the respective amine, followed by differential centrifugation. The amount of these granules was then measured. Hi, 5-HT and DA were found to be mainly localized to the granules. In a smaller proportion of the PhEA, TrpA and Eph was found there, the rest being located cytoplasmically. The present results suggest that, when taken up by rat mast cells, even amines which are endogenous to the cells are stored in the same way as the endogenous amine Hi and 5-HT.

Many authors (Purano and Green 1964, Cabot and Hagermark 1966, Jansson 1970, Frisk, Olsson and Uvnäs 1972 and Hehler and Uvnäs 1972) have shown that rat mast cells take up not only the amines which occur in them naturally—histamine (Hi) and 5-hydroxytryptamine (5-HT)—but also noradrenaline (NA) and dopamine (DA).

Granules isolated from water-lysed rat mast cells have been shown to bind various amines in a rather unselective manner (Bergendorff and Uvnäs 1973). The main constituent of the granule matrix is a heparin-protein complex (Bergqvist, Samuelsson and Uvnäs 1971) which has the properties of a weak cation exchange material. The binding sites on the complex are probably carboxyl groups (Uvnäs, Åborg and Bergendorff 1970), to which the amines are linked ionically. Since granules isolated in the above way are devoid of an intact membrane, there is no barrier to ion movements between the granules and the surrounding medium.

The object of the present study was to determine if the unselective cation storage mechanism found in granules (see above) was also operative inside the mast cell. Two separate

## References

- ABRAHAMSON, H., Reflex adrenergic inhibition of gastric motility elicited from the gastric antrum *physiol. scand.* 1974, 90 14-24.
- CHANG, P. Y. and F.-Y. Hsu, The localization of the intestinal inhibitory reflex arc. *Quart. J. exp.* 1942, 31 311-318.
- COHEN, S., L. D. HARRIS and R. LEVITAN, Manometric characteristics of the human ileocecal junction. *Gastroenterology* 1968, 54 72-75.
- GADDUM, J. H., An outflow recorder *J. Physiol. (Lond.)* 1929, 67 XI-XII.
- HIDRICHSEN, J. and A. C. IVY, Studies of the ileo-cecal sphincter in the dog. *Amer. J. Physiol.* 1949, 159 494-507.
- HULTÉN, L., Extrinsic nervous control of colonic motility and blood flow. An experimental study in the cat. *Acta physiol. scand.* 1969, Suppl. 335.
- JANSSON, G. and J. MARTINSON, Studies on the ganglionic site of action of sympathetic outflow to the stomach. *Acta physiol. scand.* 1966, 68 184-192.
- JOHANSSON, B. and J. B. LANGSTON, Reflex influence of mesenteric afferents on renal, intestinal and blood flow and on intestinal motility *Acta physiol. scand.* 1964, 61 400-412.
- JOHANSSON, B., O. JOHANSSON and B. LUNDA, Supraspinal control of the intestino-intestinal inhibitor *Acta physiol. scand.* 1963, 63 442-449.
- JOHANSSON, B., O. JOHANSSON and B. LUNDA, Tonic supraspinal mechanisms influencing the intestino-intestinal inhibitory reflex. *Acta physiol. scand.* 1968, 72 200-204.
- KELLEY, M. L. Jr, E. A. GORDON and J. A. DEWITTE, Pressure responses of canine ileocolonic junction to intestinal distention. *Amer. J. Physiol.* 1966, 211 614-618.
- KOCK, N. G., An experimental analysis of mechanisms engaged in reflex inhibition of intestinal motility. *Acta physiol. scand.* 1959, Suppl. 164.
- MARTINSON, J., Vagal relaxation of the stomach. *Acta physiol. scand.* 1965, 65 Suppl. 255.
- NAKAYAMA, S., Effect of distension of the small intestine on the movements of the gall bladder sphincter of Oddi. *Acta Med. Okayama* 1969, 23 21-26.
- PAHLIN, P.-E. and J. KEWENTER, The direct sympathetic nervous control of the cat ileo-cecal junction. *Amer. J. Physiol.* 1975, Submitted for publication.



## Intracellular Distribution of Amines Taken up by Rat Mast Cells In Vitro

By

ANDERS BERGENDORFF

Received 27 March 1975

### Abstract

BERGENDORFF A. Intracellular distribution of amines taken up by rat mast cell in vitro. *Acta physiol. scand.* 1975. 95. 133-141

At cells isolated from rat peritoneal and pleural cavities were incubated *in vitro* with radioactively labeled histamine (Hi), 5-hydroxytryptamine (5-HT), dopamine (DA), noradrenaline (NA), tyramine (T), phenylethylamine (PhEA), tryptamine (TrpA), epinephrine (Eph) or amphetamine (Amph). All these amines were taken up by the mast cells. The dose-response curves for the compound 4 $\beta$ /30-isomer release endogenous Hi and for the various amines taken up by the cells were compared. The release curves for 5-HT, DA, NA and TA were found to be similar to that for endogenous Hi, while those for PhEA, T, Eph and Amph were different from that for endogenous Hi. The uptake of Hi, 5-HT, DA, PhEA, T, Eph and Amph into granules in mast cells was studied. Membrane-bound granules were obtained by sonication of mast cells incubated with the respective amines, followed by differential centrifugation. The amine content of these granules was then measured. Hi, 5-HT and DA were found to be mainly localized to the granules, while smaller proportions of the PhEA, TrpA and Eph were found there, the rest being located in the cytoplasm. The present results suggest that, when taken up by rat mast cells, even amines which are endogenous to the cells are stored in the same way as the endogenous amine Hi and 5-HT.

Many authors (Furano and Green 1964, Cabot and Hägermark 1966, Jansson 1970, Frisk, Olsson and Uvnäs 1972 and Hekler and Uvnäs 1972) have shown that rat mast cells take up not only the amines which occur in them naturally—histamine (Hi) and 5-hydroxytryptamine (5-HT)—but also noradrenaline (NA) and dopamine (DA).

Granules isolated from water lysed rat mast cells have been shown to bind various amines in a rather unselective manner (Bergendorff and Uvnäs 1973). The main constituent of the granule matrix is a heparin-protein complex (Bergqvist, Samuelsson and Uvnäs 1971) which has the properties of a weak cation exchange material. The binding sites on the complex are probably carboxyl groups (Uvnäs, Åberg and Bergendorff 1970), to which the amines are linked ionically. Since granules isolated in the above way are devoid of an intact membrane, there is no barrier to ion movements between the granules and the surrounding medium.

The object of the present study was to determine if the unselective cation storage mechanism found in granules (see above) was also operative inside the mast cell. Two separate

approaches were used to investigate to what extent various amines, both endogenous and exogenous to rat mast cells, taken up by the cells were stored in the granules: a) Compound 48/80 is known to release the endogenous amines HI and 5-HT from mast cells in a dose-dependent manner by a process involving exocytosis of amine-containing granules. Consequently the release curves for the two amines are almost identical (Moran, Uvnäs & Westerholm 1962). This being so other amines taken up by cell ought to be released parallel with the endogenous amines if they are localized to the granules.

b) Differential centrifugation of ultrasonically disintegrated mast cells yield a mix of electron dense granules having an intact membrane, and swollen, less electron dense granules which are devoid of a limiting membrane (Anderson *et al.* 1974). The granule surrounded by an intact membrane can retain their amines even when suspended in a cation-containing medium (e.g. isotonic NaCl). This means that amines taken up by the cells and stored in the granules can be demonstrated directly.

In the present *in vitro* study all the amines investigated were taken up by the cells and stored to a greater or lesser extent in the granules.

## Methods

### Isolation of mast cells

Mixed cell suspensions were obtained from the peritoneal and pleural cavities of male Sprague-Dawley rats weighing 350–450 g and the mast cells were isolated according to the method described by Thoenes & Uvnäs (1966), as modified by Uvnäs, Åborg and Bergendorff (1970). The method was recently published in detail by Anderson *et al.* (1974).

### Isolation of granules

Isolated mast cells were sonicated (MSE, 100 Watt) in 0.34 M sucrose solution at 4  $\mu$ m for 5 s and then subjected to differential centrifugation at 350  $g$  for 10 min and 3 000  $g$  for 20 min, all procedures being carried out at 4°C. The 3 000  $g$  precipitate, containing a mixture of granules with an intact membrane and granules devoid of an intact membrane, was suspended in 0.15 M NaCl adjusted to pH 7 with Sorbo's phosphate buffer and recentrifuged (3 000  $g$  20 min, 4°C).

The intensity and duration of sonication used was found to give the largest proportion (about 30%) of granules with an intact membrane (Anderson *et al.* 1974; Anderson and Uvnäs *in press*). Sonication of the mixed granule precipitate in 0.15 M NaCl at pH 7 releases all amines stored in the granules devoid of an intact membrane by cation exchange (Åborg, Novotný and Uvnäs 1967; Uvnäs, Åborg and Bergendorff 1970; Bergendorff and Uvnäs 1971, 1973). Thus the amines remaining in the granule mixture will be localized to granules with an intact membrane.

### Charging of mast cells with amines

Isolated mast cells were pooled and suspended in a buffered salt albumin solution (154 mM NaCl, 3 mM KCl, 0.9 mM CaCl<sub>2</sub>, 10% Sorbo's phosphate buffer pH 7 and 1 mg human serum albumin/ml) hereafter called the incubation medium.

Unlabelled amine and the corresponding <sup>14</sup>C-labelled amine were added to the mast cell suspension. The concentration of mast cells was usually 0.5–0.7  $\times 10^6$  cells/ml, but was occasionally as low as 0.25  $\times 10^6$  cells/ml. The level of radioactivity was usually 1  $\mu$ Ci/ml. In those experiments in which low amine concentrations were used the radioactivity was limited to 0.5 or 0.05  $\mu$ Ci/ml. The amine concentrations used in the uptake studies varied between  $10^{-6}$  and 3 mM depending on the uptake of the amine (Fig. 1–4). In the release studies, solutions containing 0.1–0.15 mM of the amines were used, except for 5-HT and DA where concentrations of 0.005 mM and 0.02 mM respectively were used. At these concentrations the release of endogenous HI induced by compound 48/80 was not affected (Fig. 8 and 9).

The incubation of mast cells with amines was carried out in a shaking water-bath at 37°C for 90 min.

incubation, the cells were centrifuged down at 350 g for 10 min. The cells were washed in the incubation medium at room temperature until the radioactivity in the wash was close to background values (3 tubes, each of 10 ml).

#### cells

**test cells.** Cells loaded with the  $^{14}\text{C}$  labelled amine under investigation were suspended in  $10^{-4}$  M HCl and heated in a boiling water-bath for 5 min. Aliquots were taken for the assay of histamine and radioactivity (for calculations see below) and, in a few cases, for paper chromatography.

**granules.** Granules from mast cells loaded with the  $^{14}\text{C}$ -labelled amine under investigation were prepared as described above. The precipitated granules were suspended in  $10^{-4}$  M HCl and heated in a boiling water-bath for 5 min in order to release the amine stored in the granules with an intact membrane. Aliquots taken for the assay of histamine, protein and radioactivity. For calculations see below.

**histamine determination of unlabelled histamine.** Histamine was assayed by the method of Sjörs *et al.* (8) by direct condensation with o-phthalaldehyde, as described by Bergendorf and Uvnäs (1972).

**histamine determination of  $^{14}\text{C}$ -labelled amine.** The radioactivity in aliquots of test samples and the evaporated  $^{14}\text{C}$ -labelled amine incubation solution was measured using Tri-carb<sup>®</sup> liquid scintillation counter Model 3375 (Packard Instrument Co.), with Instagel<sup>®</sup> as scintillation fluid.

**histamine.** At the beginning of each experiment, aliquots of the mast cell suspension were taken for the assay of endogenous histamine and for cell counting. From these results the histamine content per  $10^6$  cells was calculated. At the end of each experiment, aliquots were taken from each sample for the determination of radioactivity and endogenous histamine. Assuming that the HI-content of intact mast cells does not change during the incubation and washing procedures (checked in 3 expts.), the number of intact mast cells at the end of the experiment was calculated from the endogenous HI-content at that time and the content per  $10^6$  cells at the start of the experiment.

Cells obtained from the above calculations enable direct comparison to be made between the amount of amine taken up by cells and by membrane-bound granules.

#### cell studies with compound 48/80

Mast cells were incubated with the  $^{14}\text{C}$ -labelled amine under investigation and washed free from external background as described above. The amine-loaded cells were exposed to different concentrations of compound 48/80 (0.1 to 30  $\mu\text{g}$  per cell), for 10 min at 37°C, and centrifuged down at 350 g for 10 min. Aliquots of the supernatant and the sediment were taken for the determination of radioactivity and histamine.

#### the paper chromatography

Ascending paper chromatography was carried out using Whatman No. 1 paper and the following solvent system: n-butanol/acetic acid/water (12:3:5). Reference chromatograms with  $^{14}\text{C}$ -labelled and unlabelled amine were run in parallel. The chromatograms of the unlabelled amine were stained with anhydrous ninhydrin. Radioactive chromatograms were cut into 1 cm strips which were cut into smaller pieces and placed in scintillation vials. 10 ml of Instagel<sup>®</sup> scintillation fluid and the radioactivity was measured as described above.

#### formation of ether-water partition coefficient

10  $\mu\text{l}$  of  $^{14}\text{C}$ -labelled amine solution (1  $\mu\text{Ci}$ ) was added to 1 ml of 0.1 M sodium phosphate buffer, pH 7.4 and 1 ml of ether saturated with this buffer. The two phases were thoroughly mixed and then allowed to separate. Aliquots were taken from each phase and the  $^{14}\text{C}$ -activity was recorded.

## Materials

histamine-(neg- $^{14}\text{C}$ ) dehydrochloride, sp. act. 39 mCi/mole; tyramine-1- $^{14}\text{C}$  hydrochloride, sp. act. 42 mCi/mole; dopamine- $^{14}\text{C}$  hydrochloride, sp. act. 39 mCi/mole; DL-noradrenaline-(carbonyl- $^{14}\text{C}$ ) hydrochloride, sp. act. 41.2 mCi/mole, The Radiochemical Centre, Amersham, England. 5-Hydroxytryptamine-2- $^{14}\text{C}$  benzoate, sp. act. 27.5 mCi/mole; tryptamine-2- $^{14}\text{C}$  benzoate, sp. act. 60 mCi/mole;  $\beta$ -phenylethylamine-1- $^{14}\text{C}$  hydrochloride, sp. act. 7 mCi/mole; New England Nuclear Boston.

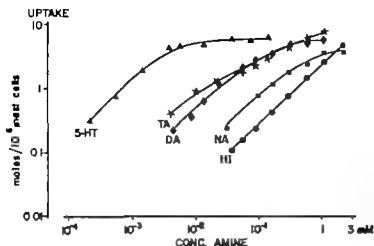


Fig. 1 Uptake by mast cells: 5-HT ( $\Delta$ - $\Delta$ ), DA ( $\odot$ - $\odot$ ), TA ( $\ast$ - $\ast$ ), NA ( $\square$ - $\square$ ), HI ( $\bullet$ - $\bullet$ ).

Mass., USA. DL Ephedrine-1 <sup>14</sup>C hydrochloride, sp. act. 20 mCi/mmol, D-amphetamine-7-<sup>14</sup>C methyl sp. act. 15.5 mCi/mmol. Commissariat à l'Energie Atomique, Gif-sur Yvette, France. Ficoil AB Pharmacia, Uppsala, Sweden, human serum albumin (free from preservatives), All Kabi, Södra, Sweden. All other substances were obtained from the usual commercial sources.

### Abbreviations

Amph, amphetamine, DA dopamine Eph, ephedrine HI histamine, 5-HT 5-hydroxytryptamine, noradrenaline PhEA,  $\beta$ -phenylethylamine TA, tyramine TrpA, tryptamine.

### Results

#### *Uptake of amines into mast cells*

All the amines investigated were taken up by isolated mast cells (Fig. 1 and 2). There were, however, large differences between the uptake curves for the different amines. None of the amines produced any HI release in the concentrations studied. Light microscopic observations did not reveal any morphological changes in the mast cells.

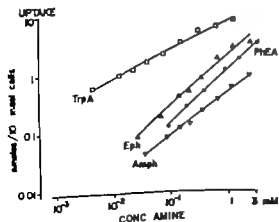
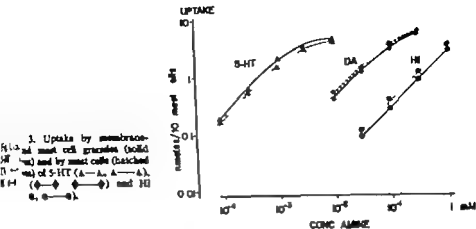


Fig. 2 Uptake by mast cells of TrpA ( $\circ$ - $\circ$ ), Eph ( $\Delta$ - $\Delta$ ), PhEA ( $\square$ - $\square$ ) and Amph ( $\circ$ - $\circ$ ).

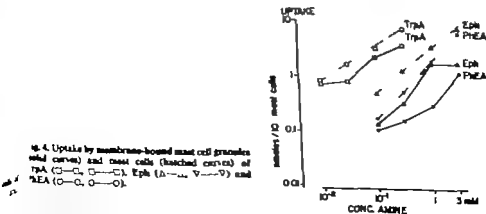


### Localization of amines

take into mast cell granules. All amines studied were found to be localized to some extent in the granules (Fig. 3 and 4). The results indicate that HI, 5-HT and DA are mainly aligned to the granules (Fig. 3), while a smaller proportion of the tryptamine (TrpA), norepinephrine (Eph) and phenylethylamine (PhEA) (Fig. 4) taken up by the cells was found in the granules.

**Amine release by compound 48/80** The compound 48/80-induced release of  $^3\text{H}$  HI and 5-HT from the mast cells occurred parallel to the release of endogenous HI (Fig. 5). Similar results were obtained for DA, NA and tyramine (TA) (Fig. 6). On the other hand, the release of PhEA, TrpA, Eph and amphetamine (Amph) (Fig. 7) did not run parallel to the release of endogenous HI. The percentage release of the latter amines induced by compound 48/80 was considerably less than the HI release.

The HI-releasing effect of compound 48/80 was found to be influenced by the concentration of DA used to load the mast cells with the amine (Fig. 8). Incubation in 0.02 mM



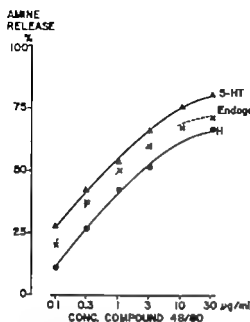


Fig. 5. Release of  $^{14}\text{C}$  labelled 5-HT ( $\Delta$ — $\Delta$ ) and HI ( $\bullet$ — $\bullet$ ) and endogenous HI (—) induced by compound 48/80.

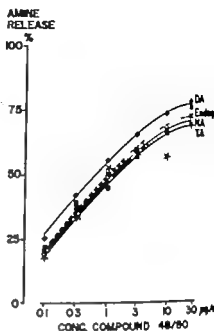


Fig. 6. Release of  $^{14}\text{C}$ -labelled DA ( $\blacklozenge$ — $\blacklozenge$ ), NA ( $\blacksquare$ — $\blacksquare$ ) and TA ( $\star$ — $\star$ ) and endogenous HI (—) induced by compound 48/80.

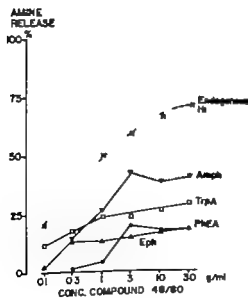


Fig. 7. Release of  $^{14}\text{C}$  labelled Amph ( $\nabla$ — $\nabla$ ), TrpA ( $\square$ — $\square$ ), PhEA ( $\circ$ — $\circ$ ) and Eph ( $\triangle$ — $\triangle$ ) and endogenous HI (—) induced by compound 48/80.

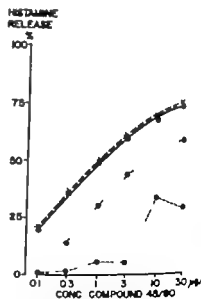
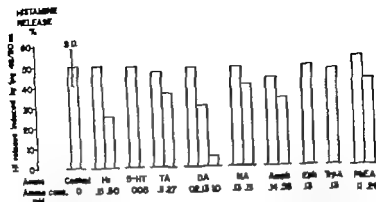


Fig. 8. Compound 48/80-induced HI release from mast cells after incubation of the cells in the presence of 0.13 mM ( $\oplus$ — $\oplus$ ), 0.13 mM ( $\circ$ — $\circ$ ) and 1.00 mM ( $\square$ — $\square$ ) DA, control HI release (—).



9 Release of endogenous HI by compound 48/80 (1 µg/ml) after incubation of the mast cells in 5-HT TrpA (same concentrations), Hs, TA, NA, Amph, Eph (two concentrations) and in DA (3 concentrations). S.D., standard deviation.

did not affect the compound 48/80-induced HI release. When higher concentrations of were used the HI-release was depressed (0.13 mM DA) or almost abolished (1 mM DA). The other amines produced analogous effects (Fig. 9). These findings were taken into consideration when choosing the amine concentration used to load the cells in the study of the release induced by compound 48/80.

#### Thin layer chromatography

Thin layer chromatograms showed only one peak of radioactivity which coincided with the peak of the radioactive reference and the ninhydrin-stained spot on the unlabelled reference chromatogram. The radioactivity levels in the samples were too low to allow reliable calculations of the percentage radioactivity found in the peak to be made. The results showing only one peak for the amines chromatographed (HI, 5-HT, PheA, TA and DA) indicate that little or no degradation of the amines took place during the experiments.

#### Octanol/water partition coefficient

Octanol/water partition coefficients for the amines were compared with that of HI, which was found to be the most water-soluble of the amines studied (Table I).

### Discussion

Isolated mast cell granules have been shown to store various cations (amines, sodium and calcium) rather unselectively by ionic binding (Bergendorff and Uvnäs 1973). HI and 5-HT are the only amines which occur naturally in rat mast cells. It was considered important to find out if amines which do not normally occur in rat mast cells could be stored in granules inside the cells.

When mast cells are exposed to compound 48/80 in a cation-containing medium, the endogenous amines HI and 5-HT are simultaneously released from the granules after excitation.

TABLE I. Ether/water partition coefficients of various amines compared to that of histamine at pH 7. The compounds are listed in decreasing order of polarity

Amine	Ether/water partition coefficients relative to HI at pH 7.4
Histamine	1
Dopamine	2
5-hydroxytryptamine	4
Noradrenaline	4
Tyramine	5
Phenylethylamine	23
Tryptamine	31
Ephedrine	47
Amphetamine	60

cytosis in exchange for cations in the extracellular medium. As the storage and release processes for HI and 5-HT are similar an amine which is taken up by the mast cells, transported into the granules and ionically bound there like HI and 5-HT ought to be released in parallel with the endogenous amines when the cells are exposed to compound 48/80. Such parallel release was found for some of the amines investigated (HI, 5-HT, DA, NE, TrpA) which suggests that they are stored like HI in the mast cell. On the other hand the release curves for PhEA, Eph and Amph did not run parallel to that of endogenous HI. This finding may indicate that only a minor part of these amines is stored in the granules, as the release of these amines is less than the release of endogenous HI at the same concentration of compound 48/80. However it is still somewhat dependent on the concentration of the releaser. The major part of these amines may be located extracellularly. More direct evidence that an amine is transported into the granules can be obtained by studying the amine content of membrane-bound granules. Good agreement was found between the cellular and granular uptakes for 5-HT, DA and HI. No such correlation was found for TrpA, PhEA and Eph, the calculated uptake into granules being less than the cellular uptake. These results, like those of the release experiments, suggest that these amines are also localized to cell sites other than granules.

The cellular uptake of 5-HT is reported to take place by active transport, that of DA and NA by facilitated diffusion and that of HI by facilitated or passive diffusion (Cahn and Haegermark 1966, Jansson 1970, Friis Holmberg and Uvnäs 1972, Heisler and Uvnäs 1972). The use of different transport mechanisms by these amines is also reflected in the present results from the study of cellular uptake. Thus 5-HT is readily taken up by the mast cell and maximal uptake is obtained already at a concentration of 0.01 mM while to get the same HI uptake a 100-fold higher concentration of HI in the incubation medium is needed. The uptake of HI, like that of several other amines studied, did not reach a maximum value in the range of concentrations used. These differences in uptake do not reflect extracellular uptake. TrpA is more readily taken up by the cell than is HI, but a smaller proportion is found in the granules. HI and Eph have almost identical cellular uptake curves but a lower proportion of the Eph taken up by the cell is localized to the granules. A correlation was found between the granular uptake and the polarity of the various



nes (Table I). The more polar amines are mainly stored in the granules, while the less polar amines are stored there to a lesser extent. This could mean that the cellular uptake is coded by transport mechanisms in the cell membrane, while the granular uptake is mainly dependent on the physico-chemical properties of the amines. Naturally selective intracellular transport mechanisms may also exist.

In conclusion, the present results indicate that amines, both endogenous and exogenous, at mast cells, taken up by the cell, are stored in the granules to some extent.

Investigation was supported by The Swedish Medical Research Council Grant B74-14X-39-10C and from Bergvall's Stiftelse. The skilful technical assistance of Margareta Wackelin is gratefully acknowledged.

## References

1. H. E. Sjöström, C. H. J. Novotny and B. Uvnäs, Ionic binding of histamine in mast cell granules. *Acta physiol. scand.* 1967 89 552-567.
2. E. Sjöström, P. P. Rönnerman, B. A. Sjöström and B. Uvnäs, Morphology and storage properties of rat mast cell granules isolated by different methods. *Acta physiol. scand.* 1974 91 145-153.
3. E. Sjöström, P. P. Rönnerman and B. Uvnäs, Selective localization of histamine to electron dense granules in antigen stimulated sensitized rat mast cells and to denser granules isolated from isolated mast cells. An electron microscope autoradiographic study. *Acta physiol. scand.* 1975 74 65-73.
4. E. Sjöström, A. and B. Uvnäs, Storage of 5-Hydroxytryptamine in rat mast cells. Evidence for an ionic binding to carboxyl groups in a granule heparin-protein complex. *Acta physiol. scand.* 1977 103 329-331.
5. E. Sjöström, A. and B. Uvnäs, Storage properties of rat mast cell granules in vitro. *Acta physiol. scand.* 1975 87 213-222.
6. E. Sjöström, U. G. Sjöström and B. Uvnäs, Chemical composition of basophil granules from isolated mast cells. *Acta physiol. scand.* 1973 83 362-372.
7. J. M. and G. H. Sjöström, Uptake, storage and release of histamine by rat peritoneal mast cells in vitro. *Acta physiol. scand.* 1966 68 206-214.
8. J. M. and G. H. Sjöström, The influence of chlorpromazine on the uptake of biogenic amines by rat mast cells in vitro. *Acta physiol. scand.* 1972 86 1-11.
9. J. M. and G. H. Sjöström, Uptake of biogenic amines by mast cells of the rat. *J. Physiol. (Lond.)* 1964 197 263-271.
10. E. Sjöström, S. and B. Uvnäs, In vivo studies on the uptake of biogenic amines by rat mast cells. *Acta physiol. scand.* 1972 86 145-154.
11. E. Sjöström, S. E. Uptake of 5-hydroxytryptamine by rat peritoneal mast cells in vitro. *Acta physiol. scand.* 1970 78 484-492.
12. J. M. and G. H. Sjöström, O. H. N. J. Rönnerman, A. L. Fark and R. J. Randall, Protein concentration with the Folin phenol reagent. *J. Biol. Chem.* 1951 193 265-275.
13. E. Sjöström, N. C. B. Uvnäs and B. WINTERHOLM, Release of 5-hydroxytryptamine and histamine from rat mast cells. *Acta physiol. scand.* 1962 36 26-41.
14. E. Sjöström, P. A. A. Sjöström and V. H. Chubb, J. A method for the fluorometric assay of histamine. *J. Pharmacol. exp. Ther.* 1959 127 182-186.
15. E. Sjöström, I. L. and B. Uvnäs, Mode of storage of histamine in mast cells. *Acta physiol. scand.* 1966 100 455-470.
16. E. Sjöström, B. C. H. Åberg and A. Sjöström, Storage of histamine in mast cells. Evidence for an ionic binding of histamine to protein carboxylate in the granule heparin-protein complex. *Acta physiol. scand.* 1970 72 Suppl. 334, 1-26.

# Noradrenaline Release Following Nerve Stimulation and its Modification by Prostaglandin $E_2$ in Human and Rabbit Oviduct

By

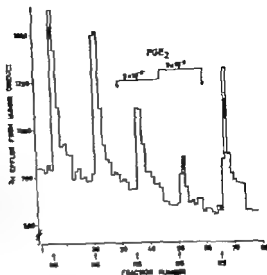
ATEF MOAWAD PER HEDQVIST and MARC BYGDEMANN

The oviduct muscular wall at the isthmus and especially at the ampullary isthmic junction (AIJ) is heavily innervated with adrenergic fibres in all mammalian species studied, including man (Brundin 1965, Öwman *et al.* 1967). The importance of the neuromuscular mechanism in this region in the physiology of ovum transport has been the subject of many studies. Locking of the fertilized ovum at the AIJ for 48-72 h is said to be governed by adrenergic control mechanisms (Pauerstein 1974). Moreover prostaglandins (PGs) have been implicated in the modification of the physiological phenomena of this organ (Spiller 1974). The effects of PGs on the release of noradrenaline (NA) have been extensively studied in other organs (*cf.* Hedqvist 1974, Frame and Hedqvist 1975). The subject of this report is the study of the effects of nerve stimulation on the neurotransmitter release, its modification by prostaglandins and the parallel effector responses in oviductal isthmus at the AIJ in rabbit and the human.

The isthmus part of human and rabbit oviducts, 1½-2 cm in length, was preincubated for one hr in Tyrode's solution containing  $2.5 \mu\text{Ci/ml}$   $^3\text{H}$ -(-)-NA (sp. act.  $5 \text{ Ci/mmol}$ , N.E.N.). It was then placed in a 2 ml bath in a longitudinal orientation and was superfused with Tyrode at a rate of 1.5 ml/min. The composition of the Tyrode was (conc. in mM): NaCl 136.7, KCl 2.7,  $\text{CaCl}_2$  1.8,  $\text{MgCl}_2$  1.0,  $\text{NaHCO}_3$  11.9,  $\text{NaH}_2\text{PO}_4$  0.4, glucose 5.5, ascorbic acid 0.1 and it was kept at  $37^\circ\text{C}$  and was bubbled with 5%  $\text{CO}_2$  in  $\text{O}_2$ . The organ was transmurally stimulated by means of platinum wire electrodes on the wall of the bath and a Grass S4 stimulator delivering trains of biphasic pulses (5 Hz, 1 ms, supramaximal voltage) at 10-15 min intervals. Contractions were recorded by an isotonic transducer (Harvard 354) loaded with 0.5-1.0 g, and a Grass Model 5 polygraph. The superfusate was drawn into one minute samples and the radioactivity was determined by counting 1 ml aliquots in a Beckman liquid scintillation spectrometer using 10 ml Instagel as counting medium. Quenching was assessed by internal standards. Prostaglandins were kindly supplied by Dr John Pike, Upjohn Co, Kalamazoo, U.S.A.

Transmural stimulation of superfused human and rabbit oviducts for 30-60 s periods (5 Hz, 1 ms, supramaximal voltage) consistently caused a marked and well reproducible increase in the release of tracer the bulk of which consisted of intact  $^3\text{H}$ -NA. The stimulation parameters, as well as the finding that tetrodotoxin (0.2  $\mu\text{g/ml}$ ) and guanethidine (2  $\mu\text{g/ml}$ ) prevented the stimulation induced release of tracer and the resultant effector response indicate activation of adrenergic nerve fibres.

Independent of hormonal condition, pregnancy estrus or progesterone dominance, the effector response to transmural nerve stimulation in the rabbit oviduct was consistently that of contraction. In the human oviduct, however the effector response was variable, i.e. contraction, no response or even marked relaxation. Presumably and in contrast to



human oviduct previously loaded with  $^3\text{H}$ -NA. Effect of PGE<sub>2</sub> on the outflow of  $^3\text{H}$ -NA in response to transmural stimulation 20 pulses at 5 Hz. Time in minutes is indicated.

this is related to the hormonal condition of the subject, since it has already been shown that estrogen predominance enhances  $\alpha$ -adrenergic receptor activity while promotes that of the  $\beta$ -receptors (Moezawi and Kim 1974).

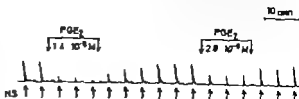
Under hormonal conditions in the rabbit as well as in the human, PGE<sub>2</sub> in doses ranging from  $9 \times 10^{-6}$  M to  $9 \times 10^{-5}$  M inhibited the release of  $^3\text{H}$ -NA in response to transmural stimulation. The degree of inhibition was dose-dependent and the effect clearly reproducible (Fig. 1).

In the rabbit oviduct of both estrogen and progesterone dominated animals PGE<sub>2</sub> caused depression of spontaneous motility. The contractions resulting from transmural nerve stimulation were also consistently reduced. The depression of both the spontaneous activity and nerve stimulation responses were dose-dependent as well (Fig. 2).

In the human, in spite of the consistent inhibition of  $^3\text{H}$ -NA release, PGE<sub>2</sub> modified the uterine motility in various ways. While spontaneous motility when present, always was markedly depressed or abolished, contractions resulting from nerve stimulation were either slightly depressed, not affected or even markedly enhanced. The one experiment where this latter effect is well demonstrated is shown in Fig. 3. This particular trace was obtained from a subject in the immediate pre-ovulatory period. Presumably therefore, her estrogen production was at a good level in conjunction with insignificant progesterone levels.

When the effector response to transmural nerve stimulation was that of depression, the inhibitory influence by PGE<sub>2</sub> should not be settled because of the almost complete cessation of

Fig. 2. Effect of PGE<sub>2</sub> on the tonic contractions of rabbit oviduct. Transmural nerve stimulation (NS) 50 pulses at 5 Hz is delivered at 5 min intervals.



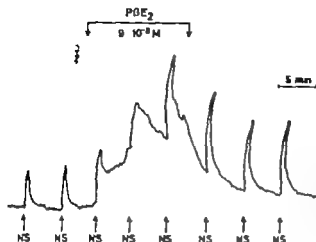


Fig. 3. Effect of  $PGE_2$  on the isometric contractions of a human oviduct in the follicular phase. Transversal stimuli (NS) 300 pulses at 5 Hz are given 5 min intervals.

spontaneous activity in the presence of  $PGE_2$ . However in no case, was the released nerve stimulation converted into one of contraction.

### Comment

The isthmus region of human and rabbit oviduct is heavily innervated with adrenergic neurons. We have shown in these experiments that nerve stimulation results in increased release in both species. The invariable inhibition of this NA release by the addition of  $\mu$ -doses of  $PGE_2$  is similar to results obtained from other adrenergically innervated tissues (cf Hedqvist 1974, Frame and Hedqvist 1975).

In the rabbit oviduct there was a parallelism between restriction of transmitter release and inhibition of effector response. The finding that nerve stimulation induced contractions of the human oviduct sometimes were enhanced by  $PGE_2$ , in spite of depressed transmitter release, is unexpected. It points out the importance of species differences. Also, the response in the human oviduct might well be related to the hormonal background of the subject. Further detailed studies correlating the various responses to the hormone levels are therefore essential.

This study was supported by grants from the Swedish Medical Research Council, project 04X-6. A.M. is on sabbatical leave from University of Chicago, supported by the Rockefeller Foundation.

### References

- BRUNDEN, J. Distribution and function of adrenergic nerves in the rabbit Fallopian tube. *J. physiol. scand.* 1965, 66 Suppl. 259 1-57.
- FRAME, M. H. and P. HEDQVIST. Evidence for prostaglandin mediated prejunctional control of vascular sympathetic tone. *Brit. J. Pharmacol.* 1975, 54 189-196.
- HEDQVIST, P., Effect of prostaglandins and prostaglandin synthesis inhibitors on norepinephrine release from vascular tissue. In *Prostaglandin Synthetase Inhibitors*, eds. Robinson, J. L. and Vane, J. R., pp. 303-309. Raven Press, New York, 1974.
- MOAWAD, A. H. and M. KIM, Plasma sex steroid levels and the *in vitro* response of the isthmus part of human oviduct to catecholamines. *Gynec. Invest.* 1974, 5 19-27.
- OWMAN, C. H., E. ROSENBERG and N. O. SJÖBERG. Adrenergic innervation of the human female reproductive organs: a histochemical and chemical investigation. *Obstet. Gynecol.* 1967, 30, 1773.
- PAUERSTEIN, C. J. *The Fallopian Tube: A Reappraisal*. Lea and Febiger, Philadelphia, 1974.
- SMILLMAN, C. H., Oviduct response to prostaglandins. Influence of estradiol and progesterone. *Prostaglandins* 1974, 7 465-469.

## Cardiopulmonary Blood Volumes at Rest and during Muscular Exercise Measured by $^{125}\text{I}$ -In Radiocardiography

By

J. KUKKA, T. PYÖRÄLÄ, P. LEHTOVIITA and A. REKONEN

Received 18 March 1975

### Abstract

KUKKA, J., T. PYÖRÄLÄ, P. LEHTOVIITA and A. REKONEN. *Cardiopulmonary blood volumes at rest and during muscular exercise measured by  $^{125}\text{I}$ -In radiocardiography* Acta physiol. scand. 1975. 95. 145-152.

Cardiac output, stroke volume, right heart and left heart blood volumes, pulmonary blood volume and aortic blood flow were measured at rest and during muscular exercise in 14 healthy subjects in the supine position. The method used was  $^{125}\text{I}$ -In radiocardiography measured precordially with a single detector. The results were analyzed with a microcomputer by modified gamma function fitting method. There was an increase in cardiac output during exercise was 140 per cent ( $p < 0.001$ ). The stroke volume increased 10 per cent ( $p < 0.05$ ) in subjects with heart rate  $< 140$  beats/min, and decreased in subjects with heart rate  $> 140$  beats/min. The right heart blood volume decreased from 115 ml/m<sup>2</sup> to 105 ml/m<sup>2</sup> (5 per cent;  $p < 0.01$ ) and the left heart blood volume decreased from 120 ml/m<sup>2</sup> to 110 ml/m<sup>2</sup> (5 per cent;  $p < 0.05$ ). The total heart blood volume thus decreased from 235 ml/m<sup>2</sup> to 215 ml/m<sup>2</sup> (5 per cent;  $p < 0.05$ ). The pulmonary blood volume showed corresponding increase from 250 ml/m<sup>2</sup> to 280 ml/m<sup>2</sup> (12 per cent;  $p < 0.05$ ), and the cardiopulmonary blood volume did not markedly change (485 ml/m<sup>2</sup> and 495 ml/m<sup>2</sup>). Aortic blood flow thus decreased from 5.0 to 2.4 (52 per cent;  $p < 0.001$ ).

Key words: Radiocardiography,  $^{125}\text{I}$ -In, cardiac output, stroke volume, heart, cardiopulmonary blood volume, muscular exercise.

Cardiac dynamic during exercise has attracted great interest. The variables measured have mainly been parameters of cardiac output, heart rate and blood pressure. Few studies have been concerned with the cardiopulmonary pools in man, because the methods giving information about central blood volumes are often inaccurate and rather cumbersome, requiring cardiac catheterization, and the results are difficult to analyze.

Radiocardiography has been used for decades for the determination of cardiac output. Its most obvious advantage is its versatility: it is atraumatic and requires only external monitoring. In addition, radiocardiography gives information about cardiopulmonary blood pools and flow times.

Henkanen (1971) and Ishai and MacIntyre (1971) have presented a model for the analysis of radiocardiograms (RCG) with the analog computer. Digital computers are, however

more frequently available. In the present study we used a modified gamma function method, first proposed by Thompson and co-workers (1964) and recently modified by (Kuikka *et al.* 1974) for the digital minicomputer for the evaluation of the cardiopulmonary pools. At rest, radiocardiography gives the same results as other well-established techniques (Donato *et al.* 1962, Vernejoul *et al.* 1964).

Vernejoul and co-workers (1964) have used radiocardiography to study changes in cardiac output and stroke volume at rest and during muscular exercise. Their results have established the suitability of this technique in connection with exercise, too.

The purpose of the present work is to evaluate the basic results for the cardiopulmonary parameters at rest and during heavy muscular exercise. The parameters measured are cardiac output, stroke volume, right heart, left heart and pulmonary blood volumes and pulmonary mean time. All these parameters are measured simultaneously. The digital computer is used for analysis of RCG curves. The results presented here form also the basis for corresponding studies in connection with the use of contraceptive and anabolic steroids.

### Material and Methods

**Material.** 14 healthy untrained test subjects were studied, 10 women and 4 men. Their mean age was 25 years (range 19–32 years).

**Procedure.** The subjects had fasted and had not smoked for 4 h before the study and had been on the measuring table for 30 min before the study. Radiocardiography at rest was performed with legs raised on the pedals of the ergometer 10 min after radiocardiography at rest, muscular exercise started. The load during exercise was 505 kpm/min (82 W) for women and 834 kpm/min (134 W) for men. Radiocardiography during exercise was done after 5 min bicycling (corresponding to a work load of 24.6 kJ for women and 41.60 kJ for men). At the time of radiocardiography the heart rate had attained a stable level. Bicycling was continued to 12 min, when all blood samples for radiocardiography were taken. The blood pressure was measured with a sphygmomanometer immediately after RCG curve recording. The heart rate was followed continuously with a cardi tachometer (DV 4, Reet, Finland) connected to the chart recorder. Measurements were made with the subject in the supine position.

**Radiocardiography.** Radiocardiography was performed as described previously by Lehtovirta and co-workers (1972). A volume of 1 ml of  $^{125}\text{I}$  (half life 100 min, gamma ray energy 393 keV, 100–400 keV) was injected i.v. and the tracer dilution curve was registered precardially with a collimated scintillation detector connected to the pulse height analyzer. The RCG curve was recorded on a multichannel analyser (channel width 0.5 s or 0.25 s) and the counts were registered by a printer. In order to confirm the bolus delivered to the heart shoulder monitoring is important.

Blood samples were taken 7 and 10 min after the injection. One sample taken during exercise before the radiocardiography was used for determining the background level of blood radioactivity.

**Analysis of RCG curves.** Cardiopulmonary parameters (cardiac output, right heart, pulmonary and left heart blood volumes and pulmonary mean time) from the RCG curves were calculated by the modified gamma function fitting method (Kuikka *et al.* 1974).

The sum of two gamma function integrands connected by the shortest transit time,  $t_p$ , between the right and left sides of the heart (atrium and ventricle combined) are fitted to the RCG curve

$$C_{200}(t) = K_1 e^{-t/a_1 + t_p/a_2} + K_2 (1 - t_p/a_2) e^{-t/a_2 + t_p/a_1}$$

where  $a_1, a_2$  = fitting parameters  
 $K_1$  = scale factor

Index 1 refers to the right heart and index 2 to the left.

When cardiac output, CO, is determined the mean blood volume of each compartment,  $V_p$ , can be expressed with the aid of the shortest mean transit times,  $t_p$  as follows.

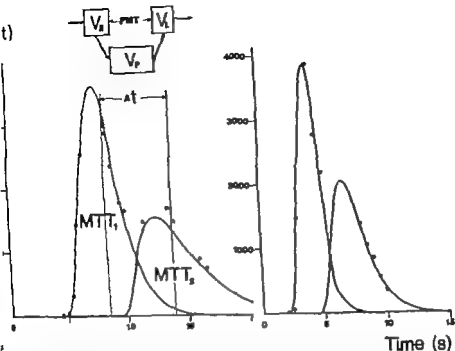


Fig. 1. Radionuclide tracer analysis of healthy female subjects at rest and during muscular exercise. The measured data are marked with open circles. Digital computer analysis of radionuclide tracer analysis: CO = 6.55 l/min at rest and 12.57 l/min, RHBV = 160 ml and 130 ml, LHBV = 150 ml and 140 ml, PSV = 520 ml and 460 ml; MTT = 4.6 and 2.2 s. The first peak of RCG curve shows the transit through the right heart and the second peak through the left.

$$V = CO \cdot \bar{t}_1 \cdot \bar{t}_2 = MTT - a_1(n), \quad (2)$$

where MTT =  $\bar{t}_1 + \bar{t}_2$  = mean transit time of tracer flow through the pool 1, and

$a_1(n) = \bar{t}_1 \sqrt{a_1(n)}$  = initial tracer distribution time of the pool (square root of variance).

$\bar{t}_2$  = pulmonary blood volume, PSV which was modified slightly for this study is obtained from the equation

$$PSV = CO \cdot \bar{t}_2 = CO \cdot \Delta t = PMV \quad (3)$$

where  $\bar{t}_2$  = pulmonary mean time

$$PMV = CO \cdot \sqrt{a_2(n) - a_1(n)} = \text{pulmonary stroke volume}$$

The cardiac output is determined in the conventional way from the RCG curve

$$CO = k \cdot \frac{A \cdot V}{A} = k \cdot 0.81 \cdot \text{equiv}/\sqrt{\text{eff}}, \quad (4)$$

where  $A = \int_0^\infty C_{RCG}(t) dt$  = area under the RCG curve (Eq. 1)

$k$  = height of the equilibrium level

$V$  = total blood volume

$k$  = correction factor

$T_{eff}$  = effective half time of tracer

The fitting of the RCG curves was performed with NOVA 1220 minicomputer by the weighted least squares method (Kearney and Clark 1970). Typical curves obtained in the analysis of the radionuclide tracer at rest and during exercise are presented in Fig. 1.

TABLE I Individual results of cardiopulmonary parameters in 14 healthy subjects at rest and during muscular exercise.

Subject	Age (years)	BSA (m <sup>2</sup> )	TBV (ml)	CO (l/min)	HR (b/min)	SV (ml/b)	RHBV (ml)	LHBV (ml)	HBV (ml)	PBV (ml)	CPBV (ml)
1 M. K. F. 23	1.55	R 3 230 E 3 240	3.90 9.30	62 145	63 85	140 130	140 130	140 130	260 270	40 50	520 510
2 L. M. F. 22	1.57	R 4 030 E 3 980	4.43 10.24	85 135	68 76	180 160	180 180	180 180	340 340	290 330	630 620
3 S. K. F. 22	1.58	R 4 180 E 4 130	5.44 11.71	75 151	70 78	220 200	230 220	230 220	450 420	330 380	780 800
4 A. N. F. 19	1.63	R 4 490 E 4 220	3.98 15.78	63 139	63 113	160 150	210 170	210 170	370 320	400 570	770 890
5 P. K. F. 21	1.66	R 3 950 E 3 690	5.43 9.40	75 123	72 77	170 180	150 130	150 130	320 280	450 500	770 780
6 M. L. F. 21	1.66	R 4 300 E 4 170	5.44 14.90	53 133	99 112	180 210	230 210	230 210	410 420	650 660	1060 1060
7 J. V. M. 32	1.76	R 5 110 E 4 630	5.50 10.75	69 138	80 78	190 170	220 210	220 210	410 380	520 530	930 910
8 P. K. M. 25	1.89	R 4 960 E 4 770	5.77 15.12	62 147	93 103	200 200	220 220	220 220	420 420	520 570	940 990
9 A. T. M. 27	1.89	R 5 330 E 5 40	6.44 14.02	72 142	87 99	290 270	240 240	240 240	530 510	490 510	1020 1020
10 P. L. M. 32	2.06	R 7 430 E 7 150	7.00 14.70	57 111	123 132	290 300	300 300	300 300	590 600	470 570	1060 1170
11 T. P. F. 21	1.50	R 4 970 E 4 860	6.88 14.57	85 165	81 76	160 130	150 140	150 140	310 270	520 440	830 730
12 P. P. F. 19	1.56	R 3 670 E 3 310	5.84 10.43	70 160	83 66	230 180	70 220	70 220	500 400	580 360	1080 760
13 S. K. F. 21	1.57	R 3 370 E 3 290	8.50 9.45	81 185	105 51	150 150	150 120	150 120	300 270	570 370	870 640
14 L. H. F. 19	1.57	R 4 230 E 4 010	8.17 14.06	100 173	82 82	210 160	180 160	180 160	390 370	470 480	860 800

F=female, M=male, R=at rest, E=during muscular exercise, BSA=body surface area, TBV=total blood volume, CO=cardiac output, HR=heart rate, SV=stroke volume, RHBV=right heart blood volume, LHBV=left heart blood volume, HBV=heart blood volume, PBV=pulmonary blood volume, CPBV=cardiopulmonary blood volume, PMT=pulmonary mean time

## Results

The individual results for measured parameters at rest and during muscular exercise are presented in Table I. The average values for the most important parameters, cardiac output, heart rate, stroke index, peripheral resistance, right heart, left heart and pulmonary blood volume, both pulmonary mean time are shown in Fig. 2.

For normalization the volume values, the ratio of these volumes to the total blood volume was calculated in per cents. The mean right heart, pulmonary and left heart blood volume at rest were  $(4.3 \pm 0.6)\%$ ,  $(9.4 \pm 2.6)\%$  and  $(4.5 \pm 0.5)\%$  and during exercise  $(4.3 \pm 0.6)\%$ ,  $(11.0 \pm 2.7)\%$  and  $(4.4 \pm 0.5)\%$ . Correspondingly the mean cardiopulmonary blood volume was  $(18.2 \pm 2.8)\%$  and  $(19.7 \pm 2.9)\%$ .

In 5 subjects radiocardiography at rest was repeated after an interval of 15 min. The individual values for cardiopulmonary parameters are found in Table II.

In Table I the cases where the heart rate during exercise was equal to or greater than 160 beats/min are italic. When divided into 2 groups (1 heart rate  $\geq 160$  beats/min)



Fig. 2a

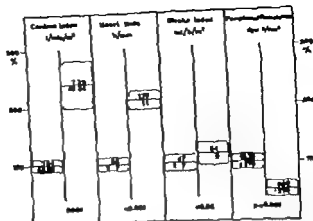
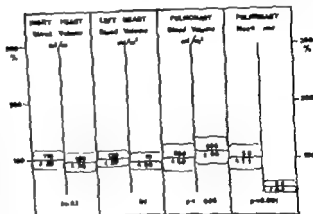


Fig. 2b



2. Mean values and standard errors of cardiopulmonary parameters of 10 healthy subjects (age 1, see text) at rest and during muscular exercise. The scale is in the change of cardiovascular parameters in per cent.

at rest 160 beats/min) the mean change in the stroke volumes between rest and exercise in group 1 is 15% and in group 2 22%.

The mean arterial pressure (MAP) was  $(98 \pm 5)$  mm Hg at rest and  $(121 \pm 7)$  mm Hg during muscular exercise. This increase was statistically highly significant ( $p < 0.001$ ). The corresponding systolic pressure was  $(123 \pm 7)$  mmHg and  $(162 \pm 4)$  mm Hg ( $p < 0.001$  for difference) and diastolic pressure  $(78 \pm 3)$  mm Hg and  $(90 \pm 14)$  mm Hg ( $p = 0.05$  for difference).

### Discussion

The cardiac index and stroke index at rest are within the limits presented by many others (Donato *et al.* 1964, Levinson, Pacifico and Frank 1966, Vernejoul *et al.* 1964). The rest values for cardiopulmonary blood volumes agree well with those presented by Perissaki and Hestenes (1971) and Ishii and MacIntyre (1971).

During muscular exercise the mean increase in cardiac output was 140% in the group 1. This increase is of the same order of magnitude as found by Bevegard, Freydrick and Strandell (1966) with a corresponding exercise load. Vernejoul and co-workers (1964) used

TABLE II Cardiopulmonary parameters of duplicate determinations in 5 healthy female subjects

Subject		HR (b/min)	CO (l/min)	RHBV (ml)	LIHBV (ml)	PBV (ml)	PMV (ml)	PMT (s)
M	I	61	4.28	190	180	290	70	4.1
	II	63	4.62	170	190	280	70	3.6
K. V.	I	81	5.01	170	180	250	100	3.8
	II	83	4.95	190	170	180	60	2.2
S. P.	I	32	5.28	250	220	330	60	3.8
	II	30	5.54	240	210	430	130	4.6
R. K.	I	79	7.26	290	250	670	210	3.3
	II	76	7.87	280	250	530	210	4.2
A. S.	I	96	7.35	200	170	400	120	3.3
	II	96	8.16	200	190	430	100	3.2
Mean	I	74	5.92	220	200	390	110	3.9
	II	73	6.22	220	200	380	110	3.6

I = first determination, II = second determination, HR = heart rate, CO = cardiac output, RHBV = right heart blood volume, LIHBV = left heart blood volume, PBV = pulmonary blood volume, PMV = pulmonary mixing volume, PMT = pulmonary mean time

lighter exercise (18 kJ) which is reflected in the smaller increase of heart rate (mean, from 72 to 92 beats/min). The increase in cardiac output was correspondingly only 40%.

There is commonly held to be a 10–20% increase in the stroke volume during exercise in untrained subjects (Bevegård, Freyschuss and Strandell 1966, Vernejoúl *et al.* 1969). In the group 1 (heart rate < 160 beats/min) the mean increase (15%) is within the traditional limits. The results in group 2 indicate that when the heart rate exceeds a certain limit (> 160 beats/min) the stroke volume begins to decrease, as reported for instance by Færevold and co-workers (1971).

There was a mean decrease of 5 per cent in the right heart and in the left heart blood volume. The total heart blood volume thus decreased (5%), a finding which is in line with the reports that radiographic heart volume decreases slightly during exercise (Marshall and Shepherd 1968). The decrease of the heart blood volume is assumed to occur mainly in the ventricles and these ventricular reductions are inversely related to ejection fractions.

The pulmonary blood volume increased 12 per cent (30 ml/m<sup>2</sup>) in group 1. Loepker and co-workers (1971) report similar increase of pulmonary blood volume during maximal exercise. In their studies these authors injected 2 indicators simultaneously into pulmonary artery and left atrium. 3 hemodynamically normal subjects were studied in all three, and there was a significant increment in pulmonary blood volume in exercise (mean 64 ml/m<sup>2</sup>). In subjects with heart rate > 160 beats/min during exercise had a mean decrease of 20 per cent in pulmonary blood volume.

The decrease in pulmonary mean time averaged 52 per cent. If pulmonary mean time is calculated in heart beats (pulmonary blood volume/stroke volume), there is no difference between the values at rest ( $5.3 \pm 1.1$  beats) and during exercise ( $5.3 \pm 0.9$  beats), suggesting a constant proportionality between pulmonary blood volume and stroke volume. There was also a highly significant correlation ( $r = +0.85$ ,  $P < 0.001$ ) between the change in stroke

size and the change in pulmonary blood volume. Therefore it seems that the pulmonary volume plays a role in the regulation of stroke volume.

The dispersive part of pulmonary blood volume (Eq. 3), mean pulmonary mixing volume was  $80 \text{ ml/m}^2$  at rest and  $60 \text{ ml/m}^2$  during exercise. This dispersion volume across lungs averaged 25 per cent less during exercise than at rest. Knopp and Bassingthwaite (1969) have found a similar decrease in dispersion with increased cardiac output in the mongrels. The lung capillaries have low compliance and it is quite likely that some previously closed pathways open when total blood flow is increased by increasing perfusion pressure (Lem, Permutt and Miseri 1968). This explanation is also compatible with our observation of increased mixing volume at long pulmonary mean time.

The increase in the blood pressures and heart rate, and the decrease in the peripheral resistance (48%) and blood volume (5%) are all within the accepted physiological limits (Hall and Shepherd 1968).

The present work gives for the first time the most important cardiopulmonary flow and volume parameters measured simultaneously both at rest and during muscular exercise. A noninvasive radioisotope method is used. The experimental verification of the parameters by invasive catheterization methods is not performed, but comparison with earlier separate works gives a close agreement as here is presented. The investigation is easy, is harmless to subject, gives reliable results and takes only 1/2 h for the measurement and analysis.

Repeatability of repeating measurements do not normally deviate more than 15 per cent (Table 1). The method is thus suitable for the study of cardiopulmonary parameters in a large group of subjects in different situations and it is also suitable for clinical use.

This investigation was supported in part by Population Council.

### References

- Knopp, S. U. FALYCHOWSKI and T. STRANDHOLM, Circulatory adaptation to arm and leg exercise in supine and sitting position. *J. appl. Physiol.* 1966, 19, 37-46.
- JOHN, P. S. PERMUTT and A. MISERI, Effect of pulmonary vascular pressures and blood flow on CO<sub>2</sub> diffusing capacity (Abstract). *Fed. Proc.* 1968, 27, 379.
- PERMUTT, L., C. GROSCHKE, M. L. LEWIS, J. DURAND, D. F. ROCHETTE, R. M. HARVEY and A. COURMANT, Quantitative radiocardiography (I), (II), (III). *Circulation* 1962, 26, 174-199.
- HELMER, J. A. B. E. ROBERTS, L. R. ELLI and J. CORWAY, Cardiovascular responses to submaximal and maximal effort cycling and running. *J. appl. Physiol.* 1971, 30, 457-461.
- BRADY, T. Analogous model for the analysis of radiocardiograms. *Circulation Res.* 1971, 5, 268-276.
- ELL, Y. and W. J. MACINTYRE, Analytical approach to dynamic radioisotope recording. *J. Nucl. Med.* 1971, 12, 793-799.
- KNOPP, T. J. and J. B. BASSINGTHWAITE, Effect of flow on cardiopulmonary transport functions. *J. appl. Physiol.* 1969, 27, 36-43.
- KUUKA, J., P. LINTONEN, E. KATKA and A. RIKONEN, Application of the modified gamma fraction for the calculation of cardiopulmonary blood pools in radiocardiography. *Phys. med. Biol.* 1974, 19, 692-700.
- LINTONEN, P., J. KUUKA, A. RIKONEN and T. PYÖLLI, Haemodynamic alterations produced by changes in pressure - with special reference to the usefulness of isotope <sup>125</sup>I in radiocardiography. *Ann. Clin. Res.* 1972, 4, 285-293.
- BRADY, G. R., A. D. FACHIO and M. J. FRANK, Studies of cardiopulmonary blood volume. Measurement of total cardiopulmonary blood volume in normal human subjects at rest and during exercise. *Circulation* 1966, 33, 347-356.

- LUEPKER, R. B., LIANDER, M., KORHONEN and E. VARNAUSKAS, Pulmonary intravascular and extracardiac fluid volumes in exercising cardiac patients. *Circulation* 1971 44 625-637.
- MARSHALL, R. J. and J. T. SHEPHERD In: *Cardiac function in health and disease* W. B. Saunders Co 1968, 31-65.
- PERÄLÄ, J. and T. HEIKKANEN, Right heart, pulmonary and left heart blood volumes determined by analogue computer analysis of radiocardiograms in normal subjects and patients with mitral stenosis. *Cardiovascular Res.* 1971 5 260-67.
- STARMER, C. F. and D. O. CLARK, Computer computations of cardiac output using the gamma variate. *J. appl. Physiol.* 1970, 28 219-220.
- THOMPSON H. K., C. F. STARMER, R. E. WHALEN and H. D. MCINTOSH, Indicator transit time considered as a gamma variate. *Circulat. Res.* 1964 14 502-515.
- DE VERNEJOUL, P., B. DELALOYE, V. DI GREGORIO and C. KELLERSHOHN, Mesure du débit cardiaque des volumes ventriculaires par radiocardiographie. *Rév. Franç. Étud. clin. biol.* 1964, 9, 693-715.

## Metabolic Characteristics of Fibre Types in Human Skeletal Muscle

By

B. EMMÉN, E. JAMNÖN, J. HENRIKSSON, A. W. TAYLOR and B. SALTIN

Received 24 March 1975

### Abstract

EMMÉN, B., E. JAMNÖN, J. HENRIKSSON, A. W. TAYLOR and B. SALTIN. *Metabolic characteristics of fibre types in human skeletal muscle*. Acta physiol. scand. 1975. 95. 153-165

side biopsy samples were obtained from healthy subjects in order to evaluate quantitative differences in the levels of substrate (glycogen and triglyceride) and ion concentrations ( $\text{Na}^+$  and  $\text{K}^+$ ) as well as enzyme activity levels (succinate dehydrogenase, SDH, phosphofructokinase, PFK, 3-hydroxyacyl-CoA dehydrogenase, HAD, myosin ATPase) between human skeletal muscle fibre types. After freeze drying the muscle specimen fragments of single fibres were dissected out and stained for myofibrillar ATPase with preincubation at pH's of 10.3, 4.6, and 4.35. Type I ("red") and II A, B, and C ("white") fibres could be identified. Glycogen content was the same in different fibres, whereas triglyceride content was highest in Type I fibres (2-3 X Type II). No significant differences were observed for  $\text{Na}^+$  and  $\text{K}^+$  between the types. The activity for the enzymes studied were quite different in the fibre types (SDH and HAD:  $\text{Type I} > \text{Type II}$ ; PFK:  $\text{Type I} \approx 0.5$  Type II; myosin ATPase:  $\text{Type I} \approx 0.4$  Type II). The subgroups of Type II fibres are distinguished by differences in both SDH and PFK activities (SDH:  $\text{Type II C} > \text{A} > \text{B}$ ; PFK:  $\text{Type II B} > \text{A} > \text{C}$ ). It is concluded that contractile and metabolic characteristics of human skeletal muscle are very similar to many other species. One difference, however, appears to be that no Type II fibres are at oxidative potential higher than Type I fibres.

Since the first description of difference in colour between fibres in mammalian skeletal muscle many studies have further differentiated and characterized fibres in skeletal muscle. To some extent investigation has now reached the molecular level. However, these studies are not always focused on the characterization of the specific features of different fibre types. This is especially true for studies on human skeletal muscle where it is very difficult to obtain pure samples containing only one fibre type.

Recently Peter *et al.* (1972) described selected biochemical characteristics of the fibres found in skeletal muscles of guinea-pig and rat. They were able to differentiate three types of fibres, which they named fast twitch oxidative (red, Type II), fast twitch glycolytic (white, Type I) and slow twitch oxidative (intermediate, Type I) fibres. In most species some muscles or muscle bundles contain only one type of fibres thereby making it relatively easy to characterize biochemically each fibre type separately. In muscles where the fibre types are mixed as in man (Johnson *et al.* 1973) it is difficult to obtain quantitative

measurements of one fibre type. Such information is needed, however to enable quantitative biochemical characterization of human muscle fibre types, and in better to stand their metabolism and degree of adaptability.

The difficulties described above can be partially overcome by using human samples which contain known percentages of Type I and Type II fibres extrapolating biochemical analyses of both fibre type to 100% (Taylor, Eserén and Saltin 1974). However a better approach to this problem is to dissect each fibre type and in base qualitative biochemical measurements on either single or pooled fibres.

Using this technique the two main objectives of this study are to describe in detail, methods that can be used to quantify substrate and ion concentrations as well as enzyme activities levels in fibres of skeletal muscles in man, and to describe some biochemical characteristics of these fibre types.

### Material and Methods

*Material.* Approximately 150 muscle samples were taken and analysed from 35 healthy males and 10 females. The subjects differed in their background of physical activity and were 20-39 years old.

*Muscle sampling.* The muscle samples (10-50 mg) were usually obtained by a needle biopsy (Bergström 1962) and in some few cases by a surgical procedure. Before the biopsy was performed subjects were informed about the nature of the procedure and, as all of them had participated in similar types of studies before, they were well aware of both immediate and late side effects. The samples were taken at rest from the lateral portion of the thigh as well as from the deltoid, gastrocnemius and other muscles.

*Classification of fibres.* Based on histochemical stain for myofibrillar ATPase at pH 9.4 (Pacholski-Herman 1935) after preincubation at pH 10.3 and 4.3 as well as NADH-dehydrogenase, these muscles as most other skeletal muscles in man contain two major fibre types (Gollnick *et al.* 1972).

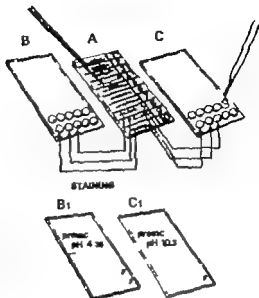
The fibres staining dark for myofibrillar ATPase at alkaline preincubation have been named Type II and the others Type I fibres (Engel 1964). The reverse staining pattern is obtained with the acid preincubation at pH 4.3, (Guth and Samaha 1969).

*Preparation of fibres.* Freeze dried material was used in order to obtain single muscle fibres. Freeze dried material absorbs  $O_2$ ,  $N_2$  and moisture when taken out of vacuum but reaches equilibrium in a few seconds (Lowry and Passonneau 1973). Absorption of approximately 5% of the fibre weight can be assumed to occur at 20°C with less than 35% humidity. Our dissection was therefore performed under such conditions. That no further change in fibre weight took place in our room was ensured by weighing the same fibres repeatedly during one day. The freeze dried material was placed under a dissection microscope (40 $\times$ ) and fragments of single muscle fibres were dissected out. The dissecting tools were constructed of wooden sticks with minute metal needles glued to one end.

The single fibres were then placed on a microscope slide, the ends cut off and placed in drops of water on another slide. After evaporation of the water the fibre ends were identified by staining for myofibrillar ATPase at pH 9.4 after preincubation at pH 10.3 for 15 min at 38°C in 0.05 M glycine buffer, 0.03 M  $CaCl_2$ , 0.05 M NaCl. The fibre ends were then stained either positively or negatively. Also the other fibre was cut off from each fibre and stained after preincubation at pH 4.3 for 5 min at room temperature in 0.06 M NaAc-0.1 M KCl. Thus gained the opposite staining pattern also for single fibres.

After the fibres were classified the remaining portions of the fibre was weighed on a quartz fibre balance that had been calibrated with quinine hydrobromide. The weights of the fibres varied from 0.5 to 5  $\mu$ g. The above procedure is schematically illustrated in Fig. 1. In addition the above staining procedure an acid preincubation at pH 4.6 was in some instances also included (Plate 3). As shown by Weidner and Kaiser (1970) this procedure allows further separation of Type II fibres in subgroups A, B and C.

It is well documented that muscles in larynx, chewing muscles, and muscles around the eyes differ in their fibre characteristics.



1 The picture illustrates schematically staining procedure of individual muscle types. The fibres are divided in three (A) and the two small ends are each in a drop of water (B, C) that is used as evaporate. The result after staining myofibrillar ATPase after different pH (C) is shown.

care in proper classification is subgroups as possible stages for NADH and -glycero-phosphate dehydrogenase were also used. These procedures called for extraordinarily long fragments of muscle fibres I could then only be performed on small number of samples.

**biochemical analysis.** As in our earlier work the biochemical methods are based on NAD-NADP dependent enzyme fluorescence (Farrall) assay.

**Triglycerides.** A single fibre (0.5-5  $\mu$ g) was placed in capillary tube and 8  $\mu$ l of 1 M HCl was added. The tube was then sealed and placed in an oven at 100°C for 2 h. From the hydrolyzed solution 2  $\mu$ l was added 150  $\mu$ l of reagent solution and analyzed for glucose residues (Eadie and Hegdickson 1974). The coefficient of variation determined on 12 divided fibres was 6.2%.

**Triglycerides.** Pooled Type I or Type II fibres from the same muscle sample (50-100  $\mu$ g) were homogenized in 8.5 ml of MeOH and 1 ml of  $\text{CHCl}_3$ . 1.5 ml of solvent was added. After 24 hours the  $\text{CHCl}_3$  phase was removed and evaporated under stream of  $\text{N}_2$ . 75  $\mu$ l of 0.05 M  $\text{E}_2\text{NOH}$  in ethanol was added and the sealed tubes were heated at 60°C for 30 min in a water bath. 75  $\mu$ l of 0.1 M HCl were then immediately added and the contents mixed. Fatty acids were removed by shaking with 2 ml of hexane. The hexane layer was taken off and an aliquot of the hydrolysate was taken for glycerol determination according to Charnick (1969). Appropriate triglyceride standards and blanks were treated in the same manner as the samples. Determination of the coefficient of variation on 6 pooled samples of the same type gave values as high as 11%. As the error of the method of the analytical procedure only was 3%, the large differences in values must likely reflect the heterogeneous storage of lipids in the muscle fibres.

**Lactate dehydrogenase (LDH EC 1.3.99.1).** From 5 to 10  $\mu$ g muscle samples (3-15 fibres of similar size from one muscle sample) were used. The pooled fibres were incubated in 75  $\mu$ l 0.3 M potassium buffer (pH 7.7) with 0.05 M BSA for 5 min at room temperature.

The sample was made 0.5 mM with regard to FMN (light sensitive), and 150 mM to Na-pyruvate and was incubated in a water bath in the dark for 30 min at 38°C. Pyruvate and malate were used as standards. The reaction was stopped with 15  $\mu$ l 1 M NaOH and 30  $\mu$ l bromocresol, mixed and centrifuged for 5 min.

A quantitative determination of the formate and malate produced was then made on the supernatant. 10  $\mu$ l were added to 1 ml of 0.1 M hydrate buffer (pH 9.0) containing 2.0 mM EDTA and 0.56 mM NAD. Blank fluorescence was read and the solution and 0.25  $\mu$ g/ml of formate and 5  $\mu$ g/ml of malate dehydrogenase added, and the reaction allowed to proceed for 2 h. The activity was expressed in nmol

fumarate or malate per kg dry weight per min. The coefficient of variation determined on 8 paired samples was 6.7%.

**Phosphofructokinase (PFK EC 2.7.1.11).** A single fibre (0.5–2  $\mu$ g) was added in 50  $\mu$ l 0.04 M Tris-HCl pH 7.6 and incubated for 2 min at room temperature.

The reaction was started with 1 ml reagent solution containing 0.04 M imidazole buffer pH 7.6,  $\text{MgCl}_2$  1 mM, ATP 5 mM, ADP 5 mM,  $\text{P}_i$  0.2 mM, 5-AMP 1 mM, G-6-P 3 mM,  $\text{NH}_4^+$  0.81 M, 1  $\mu$ g/ml phosphoglucose isomerase, 15  $\mu$ g/ml aldolase, 3  $\mu$ g/ml triphosphatase and 75  $\mu$ g/ml glyceraldehyde dehydrogenase. Fructose 1-6-diphosphate was used as standard. The test tube and fibre was shaken every second minute. The activity was expressed mmol fructose-1,6-bisphosphate produced per kg dry weight per min. The coefficient of variation for PFK determined on 10 paired samples was 6.5%.

**3-Hydroxyacyl-CoA-dehydrogenase activity (HAD EC 1.1.1.35).** Determinations were made on 10 muscle samples homogenized in 0.3 M potassium phosphate buffer (pH 7.0).

The homogenate was added to a reagent solution containing 0.05 M imidazole buffer pH 7.6, BSA, 0.1 mM EDTA, 0.05 mM NADH and 0.02 mM acetoacetyl-CoA. NADH was used as a standard with the blank being handled in the same way as the sample but without acetoacetyl-CoA. The activity was expressed as the maximal initial rate, mmol 3-hydroxyacetoacetyl-CoA produced per kg wet weight per min.

**Myosin ATPase.** Muscle samples weighing 5–15 mg were homogenized in 5 ml of 0.15 M KCl-Hisidine-0.005 M EDTA (pH 7.0). After centrifugation at 1500 g for 20 min the supernatant was discarded. The precipitate was washed in 0.15 M KCl-0.01 M Hisidine (pH 7.0) and then dissolved in 6.5 ml of 0.15 M KCl-6 mM  $\text{Na}_2\text{S}_2\text{O}_8$  (pH 7.2). The sample was then centrifuged at 100 000 g for 30 min to remove membranes and muscle residue. The supernatant was dialysed with distilled water overnight. The myosin was released from the dialysis tube by centrifugation and resuspended in 0.5 M KCl pH 7.5. All the procedures were performed at 4°C.  $\text{Ca}^{++}$  activated ATPase was assayed according to Strydom et al. (1971) in a final volume of 1 ml containing 0.05 M TRIS, 0.5 M KCl, 1 mM EDTA and 5 mM ATP. Incubation was stopped after 10 min at 25°C by adding 0.5 ml of 10% perchloric acid. The liberated  $\text{P}_i$  was measured using a scintillation counter (Rockwell and Herron (1953)). Protein was measured as described by Lowry (1951). The activity was expressed as  $\mu$ mol  $\text{P}_i$  per mg per min. The fibre composition of the sample was determined on a separate sample which was histochemically stained for myofibrillar ATPase after preincubation both at pH 7.0 and 10.0. Pooled fibres (150–200  $\mu$ g) were treated in the same way as the homogenate except that the incubation time was 15 min.

$\text{K}^+$  and  $\text{Na}^+$  concentrations were determined on single fibres (0.5–3  $\mu$ g) with an integrating flame photometer (Nilhem AB, Stockholm, Sweden). This apparatus was sufficiently sensitive to detect  $\text{Na}^+$  concentrations of  $10^{-12}$  mol, thus allowing fibre size as small as 0.5  $\mu$ g to be used. The accuracy of the method was within  $\pm 4\%$ .

0.5  $\mu$ l of  $\text{H}_2\text{O}$  was used to attach the fibre to the platinum-iridium loop. Appropriate standards containing both  $\text{Na}^+$  and  $\text{K}^+$  were used and 0.5  $\mu$ l of the solution was placed on the loop. The loop must be heated in the flame, which was ensured by the use of radiation heat for at least 1/2 minute. It was revealed that neither of the ions nor proteins interfered with the determinations.

## Results

Table I summarizes the observations made on fibres from human muscle which were stained positively or negatively for myofibrillar ATPase after alkaline preincubation. It is assumed that the difference in staining pattern corresponds to a real difference in the activity of myosin ATPase and the extrapolated values from the linear equation  $y = 0.16 + 0.48x$  ( $y$  = myosin ATPase,  $x$  = fibre composition) comes out to be 0.16 and 0.48 mmol  $\text{P}_i$  per mg myosin per min for Type I and Type II fibres, respectively (Taylor, Essén and Herron 1974). These findings are further substantiated by determinations on pooled samples of the major types resulting in 2.2–2.5 times higher ATPase activity levels in Type II than in the Type I fibres.



TABLE 1. Mean values  $\pm$  S.D. and range for certain biochemical characteristics of skeletal muscle in some number of subjects.

	Myofibrillar ATPase stain preincubated at pH 10.3	
	Positive (Type II)	Negative (Type I)
$\text{ATPase}$ $\text{mmol min}^{-1} \text{kg}^{-1}$ $\text{PUC in}$ $2$	0.48	0.16
$\text{glycogen}$ $\text{mmol kg}^{-1} \text{dw}$ $\text{PUC in}$ $617$ fibres	$359 \pm 92$ $(115-749)$	$355 \pm 140$ $(98-881)$
$\text{triglyceride}$ $\text{mmol kg}^{-1} \text{dw}$ $\text{PUC in}$ $4$ 28 samples	$74 \pm 46$ $(21-164)$	$207 \pm 86$ $(49-328)$
$\text{phosphocreatine}$ $\text{mmol kg}^{-1} \text{dw}$ $\text{PUC in}$ $12$ , 101 fibres	$40.4 \pm 7.3$ $(17.3-99.6)$	$25.8 \pm 6.9$ $(8.0-61.3)$
$\text{malate dehydrogenase}$ $\text{mmol min}^{-1} \text{kg}^{-1} \text{dw}$ $\text{PUC in}$ $16$ , 52 samples	$19.3 \pm 7.6$ $(6.7-39.5)$	$29.6 \pm 7.2$ $(22.7-50.6)$
$\text{ATPase}$ $\text{mmol kg}^{-1} \text{dw}$ $\text{PUC in}$ $2$ , 408 fibres	$632 \pm 18$ $(598-700)$	$617 \pm 21$ $(592-690)$
$\text{ATPase}$ $\text{mmol kg}^{-1} \text{dw}$ $\text{PUC in}$ $2$ : 408 fibres	$102 \pm 7$ $(89-122)$	$98 \pm 6$ $(86-115)$

Extrapolated values from linear regression (see text)

Individual fibres.

Pooled fibres.

See text.

The mean content of glycogen was 355 and 359 mmol per kg in the Type I and II fibres, respectively. The rather wide variation observed around these mean values is partly a function of different resting glycogen levels in the muscle studied. However, in most cases 5-90% of the fibres in one muscle fell within 50 mmol per kg from the mean value. The density of the PAS stain varies somewhat but at near or above normal glycogen content the muscle, the histochemical stain for glycogen content does not appear to be sensitive enough (Plate 1 and Fig. 2).

The mean triglyceride concentration is almost 3 times higher in Type I as compared to II fibres (207 vs. 74 mmol per kg d.w.). A large scatter around the mean value is also found for this substrate. This can probably be attributed to differential distribution of fat in the muscle samples. Thus a t-test on paired determinations, i.e. pooled II and I fibres from the same muscle sample, showed a highly significant difference (Fig. 2). The histochemical picture also indicates such a difference (Plate 1).

The only glycolytic enzyme studied, PFK, also demonstrated a significant difference

malate per kg dry weight per min. The coefficient of variation determined on 3 paired samples was 6.7%.

**Phosphofructokinase (PFK EC 2.7.1.11).** A single fibre (0.5–2 µg) was added to 50 µl 0.04 M imidazole buffer pH 7.6 and incubated for 2 min at room temperature.

The reaction was started with 1 ml reagent solution containing 0.04 M imidazole buffer pH 7.6, 1 mM  $MgCl_2$ , 1 mM ATP, 3 mM ADP, 5 mM  $P_i$ , 0.2 mM 5-AMP, 1 mM Cl-6-P, 3 mM  $NH_4Cl$ , 0.01 M  $NaH_2PO_4$ , 1 µg/ml phosphoglucose isomerase, 15 µg/ml aldolase, 3 µg/ml triphosphatase and 15 µg/ml glycero-phosphatide dehydrogenase. Fructose 1-6-diphosphate was used as standard. The test tube with fibre was shaken every second minute. The activity was expressed mmol fructose-1,6-bisphosphate produced per kg dry weight per min. The coefficient of variation for PFK, determined on 3 paired samples, was 6.3%.

**3-Hydroxyacyl-CoA-dehydrogenase activity (HAD EC 1.1.1.35).** Determinations were made on muscle sample homogenized in 0.3 M potassium phosphate buffer (pH 7.0).

The homogenate was added to a reagent solution containing 0.05 M imidazole buffer pH 7.6, BSA, 0.1 mM EDTA, 0.05 mM NADH and 0.02 mM acetoacetyl-CoA. NADH was used as standard with the blank being handled in the same way as the sample but without acetoacetyl-CoA. The activity was expressed as the maximal initial rate, mmol 3-hydroxyacetoacetyl-CoA produced per kg wet weight per min.

**Myosin ATPase.** Muscle samples weighing 3–15 mg were homogenized in 5 ml of 0.15 M NaCl, 0.005 M EDTA (pH 7.0). After centrifugation at 1500 g for 70 min the supernatant was discarded. The precipitate was washed in 0.15 M KCl–0.01 M Histidine (pH 7.0) and then dissolved in 0.5 ml of 0.1 M  $K_2S_2O_8$  (pH 7.2). The sample was then centrifuged at 100 000 g for 30 min to remove and muscle residue. The supernatant was dialysed with distilled water overnight. The myosin was removed from the dialysis tube by centrifugation and resuspended in 0.5 M KCl pH 7.5. All the preparations were performed at 4°C.  $Ca^{++}$  activated ATPase was assayed according to Sjöström et al. (1973). The final volume of 1 ml containing 0.05 M TRIS, 0.5 M KCl, 1 mM EDTA and 5 mM ATP. Incubation stopped after 10 min at 25°C by adding 0.5 ml of 10% perchloric acid. The liberated  $P_i$  was measured according to Rockstein and Herron (1951). Protein was measured as described by Lowry (1951). The activity was expressed as µmol  $P_i$  per mg per min. The fibre composition of the sample was determined on a fibre which was histochemically stained for myofibrillar ATPase after preincubation both at pH 7.0 and 4.3. Pooled fibres (150–200 µg) were treated in the same way as the homogenates except that the volume in which they were homogenized was 2 ml and the incubation time was 15 min.

**K<sup>+</sup> and Na<sup>+</sup> concentrations** were determined on single fibres (0.5–3 µg) with a integrating flame spectrometer (Nilham AB, Stockholm, Sweden). This apparatus was sufficiently sensitive to detect Na<sup>+</sup> concentrations of  $10^{-12}$  mol, thus allowing a fibre size as small as 0.5 µg to be used. The accuracy of the method was within  $\pm 4\%$ .

0.5 µl of  $H_2O$  was used to attach the fibre to the platinum-iridium loop. Appropriate standards containing both Na<sup>+</sup> and K<sup>+</sup> were used and 0.5 µl of the solution was placed on the loop. The loop must be dry when it is placed in the flame, which was ensured by the use of radiation heat for at least 1/2 minute. It was revealed that neither of the ions nor proteins interfered with the determinations.

## Results

Table I summarizes the observations made on fibres from human muscle which were positively or negatively for myofibrillar ATPase after alkaline preincubation. It is assumed that the difference in staining pattern corresponds to a real difference in the activity of myosin ATPase and the extrapolated values from the linear equation  $y = 0.16 + 0.48x$  ( $y$  = myosin ATPase,  $x$  = fibre composition) comes out to be 0.16 and 0.48 mmol myosin per min for Type I and Type II fibres, respectively (Taylor, Essén and Jansson 1974). These findings are further substantiated by determinations on pooled samples of the major types resulting in 2.2–2.5 times higher ATPase activity levels in Type II than in the Type I fibres.

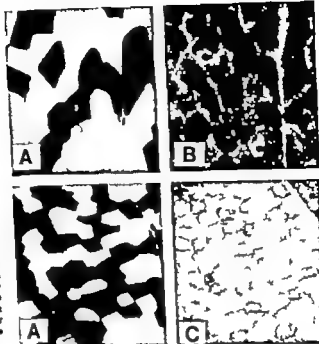


Fig. 1. Photomicrographs ( $\times 1300$ ) of vastus lateralis from one subject illustrating myofibrillar ATP-staining after preincubation at pH 10.3 (A) and the PAS-stain glycogen (B) and OIL RED stain for neutral fat (C). Notice the amount of glycogen in both types but that the stain for neutral fat is more intense in type I.

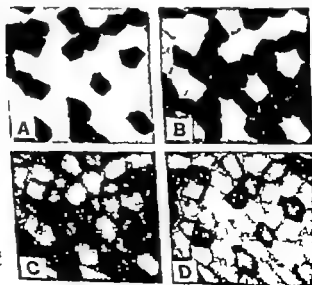


Fig. 2. Photomicrographs ( $\times 1300$ ) of vastus lateralis from one subject illustrating myofibrillar ATP-staining after preincubation at pH 10.3 (A) and pH 4.35 (B). NADH-dehydrogenase (C) and  $\alpha$ -glycerophosphate dehydrogenase (D). Notice the opposite staining pattern when comparing micrographs A and B.

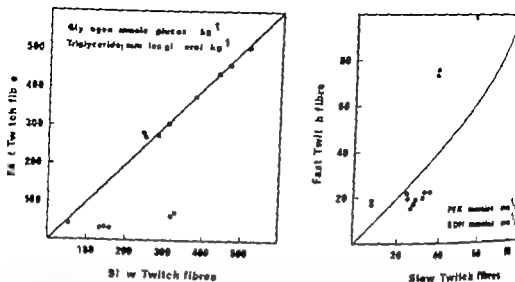


Fig. 2. The diagrams represent triglyceride and glycogen concentrations and the activity of PFK in type I (slow twitch) and type II (fast twitch) fibres from different muscle samples and mitochondria. One point shows the concentration of substrate or activity in type I (x-axis) and type II (y-axis) in the sample.

between the fibre types. In all cases where a paired comparison between fibres from same muscle could be made, the highest activity was found in the Type II fibres (Fig. 2). The stain for  $\alpha$ -glycerophosphate-dehydrogenase is often used as a marker for glycolytic potential and from Plate 2 it can be observed that there is a definite difference between Type I and II fibres and minor variations exist between fibres of the same type.

Fibres positively stained for myofibrillar ATPase (alkaline preincubation) have the highest staining intensity for NADH-dehydrogenase. As this latter stain is an indicator of oxidative potential of the fibre, there is a good agreement between the histochemical and the quantitative determinations of SDH (Plate 2 and Fig. 2). Muscle samples from 10 subjects were used to study the SDH activity in Type II A, B and C fibres as well as Type I fibres. Of these samples, three were large enough to stain not only for myofibrillar ATPase (3 different preincubations) but also for NADH and  $\alpha$ -glycerophosphate-dehydrogenase. Moreover on these three samples the PFK-activity was also determined. It appeared that within one muscle the SDH-activity in Type II A fibres is lower than the activity in Type I fibres but higher than in Type II B ( $p < 0.05$  Table II). The SDH-activity in Type II C fibres is very close to the activity in Type I fibres, but these fibres are very few (small number of fibres).

The PFK-activity differs between Type I and II fibres and when comparing the groups of Type II fibres, a somewhat higher PFK-activity appeared in II B fibres.

The activity for HAD was only evaluated on samples with a mixed fibre population (30–90% Type I fibres). The activity was approximately 2 times higher than the activity giving values between 10–20 mmol  $\times$  (kg  $\times$  min)<sup>-1</sup>. When relating the HAD activity to fibre composition a similar pattern as for SDH was observed. This may indicate a similar difference between fibres types for HAD as for SDH.

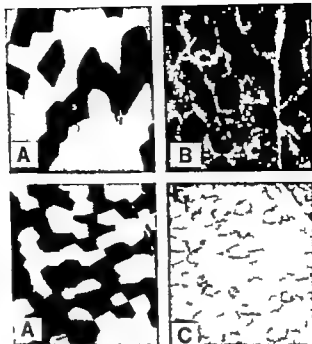


Figure 1. Photomicrographs (130 $\times$ ) of various fasciculi from one subject illustrating myofibrillar ATPase staining after preincubation at pH 10.3 (A) and the PAS-stain for glycogen (B) and OIL RED O stain for neutral fat (C). Notice equal amount of glycogen in both fiber types but that the stain for neutral fat is more intense in type I fibers.

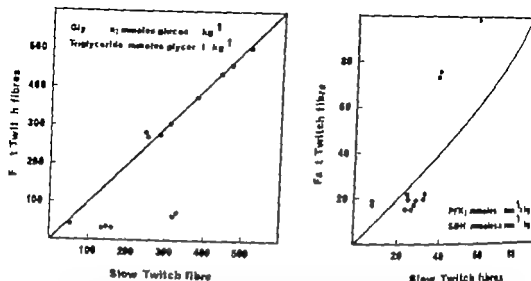


Fig. 2. The diagrams represent triglyceride and glycogen concentrations and the activity of SE and PFK in type I (slow twitch) and type II (fast twitch) fibres from different muscle samples and one point shows the concentration of substrate or activity in type I (x axis) and type II (y-axis) in the same sample.

between the fibre types. In all cases where a paired comparison between fibres from the same muscle could be made, the highest activity was found in the Type II fibres (Fig. 2). The stain for  $\alpha$ -glycerophosphate-dehydrogenase is often used as a marker for glycolytic potential and from Plate 2 it can be observed that there is a definite difference between Type I and II fibres and minor variations exist between fibres of the same type.

Fibres positively stained for myofibrillar ATPase (alkaline preincubation) have the low staining intensity for NADH-dehydrogenase. As this latter stain is an indicator of the oxidative potential of the fibre, there is a good agreement between the histochemical picture and the quantitative determinations of SDH (Plate 2 and Fig. 2). Muscle samples from 10 subjects were used to study the SDH-activity in Type II A, B and C fibres as well as Type I fibres. Of these samples, three were large enough to stain not only for myofibrillar ATPase (3 different preincubations) but also for NADH and  $\alpha$ -glycerophosphate-dehydrogenases. Moreover on these three samples the PFK-activity was also determined. It appears that within one muscle the SDH-activity in Type II A fibres is lower than the activity in Type I fibres but higher than in Type II B ( $p < 0.05$  Table II). The SDH activity in Type II C fibres is very close to the activity in Type I fibres, but these fibres are very few (<2% of total number of fibres).

The PFK-activity differs between Type I and II fibres and when comparing the two groups of Type II fibres, a somewhat higher PFK-activity appeared in II B fibres.

The activity for HAD was only evaluated on samples with a mixed fibre population (30-90% Type I fibres). The activity was approximately 2 times higher than the SDH activity giving values between 10-20 mmol  $\times$  (kg  $\times$  min)<sup>-1</sup>. When relating the HAD activity to fibre composition a similar pattern as for SDH was observed. This may indicate the same difference between fibres types for HAD as for SDH.

III. Comparison of determinations performed on muscle sample divided in two portions. One part was homogenized and the other part was freeze dried and dissected. The latter values are recalculated to wet weight based on water content of 76%.

	Homogenate	Type I	Type II	Type I+II (mean)
Glycogen mol kg <sup>-1</sup> 17	58.5	54.6	58.8	56.7
K mol kg <sup>-1</sup> mm <sup>-1</sup> 12	20.5	6.2	11.9	9.0
SDH mol kg <sup>-1</sup> mm <sup>-1</sup> 10	9.7	7.5	5.4	6.4

has been obtained. The concern by Guth and Samaha (1969) that some fibres show a false negative stain for myofibrillar ATPase due to the staining of mitochondrial ATPase is most likely not a major problem in the present study. None of the subgroups of the Type II fibres have as high oxidative capacity as Type I fibres. Therefore, including the stain for myofibrillar ATPase with the preincubation at pH 4.35 and for NADH-diaphorase minimizes a mistake in the classification (Fig. 1 and Plate 2).

To validate how representative the single fibre technique is, the following controls were performed. The muscle fibre composition was determined in six muscle samples both on cross-sections of the samples and by counting the number of Type I and II fibres ( $\approx 200$  fibres) obtained from dissection. With these two procedures the mean difference in fibre composition was 1.6%. The concentration of glycogen and the activity of PFK and SDH were determined on mixed muscles (homogenate) and individual fibres from the same muscle specimen. For glycogen an almost complete agreement was found (Table III). The PFK and SDH-activities, however, were consistently less when the determinations were performed on freeze dried material. This may have the following explanation.

Freeze dried muscle tissues can be stored in vacuum at  $-20^{\circ}\text{C}$  for several days without demonstrable change in the content of substrate or enzymes. After a day at room temperature in air substrate concentration is from a practical standpoint unaffected. However the activity of enzymes declines significantly within hours (Fig. 3). Thus when enzyme measurements are performed, the shortest possible time should elapse between the dissection and the assay. In the present study the assays were performed within 4–12 h, and PFK and SDH assay within 4–6 h. This then means that the values obtained for SDH activity are reduced approximately 50% (Fig. 3). Taking this into account there is a reasonable agreement also for the enzyme determinations.

At present we have no method to determine both wet and dry weight of the fibres, and values, except for myosin ATPase, are therefore expressed in relation to the dry weight. The portion of extracellular material around the fibres may vary slightly a determination of the true extra- and intracellular mass would be valuable. This is especially appropriate for the determinations of ion content.

TABLE II In the table is given the enzyme activities for succinate dehydrogenase (SDH) and phosphofructokinase (PFK) in different fibre types in human skeletal muscle (one subject). The measurements were performed on fragments of fibres and mean values of 3-5 fibres and the range are given. No correction for loss of enzyme activity has been made (cf. Fig. 3 and Table III).

	Type I	Type II A	Type II B	Type II C
SDH mmol $\times$ kg <sup>-1</sup> $\times$ min <sup>-1</sup>	2.5 (3.1-1.8)	1.9 (2.5-1.5)	1.0 (1.3-0.4)	2.4 (2.8-2.1)
PFK mmol $\times$ kg <sup>-1</sup> $\times$ min	3.9 (4.9-2.1)	5.6 (6.4-3.0)	6.9 (8.3-4.5)	5.5 (5.9-5.0)

The potassium concentration averaged 617 and 634 mmol  $\times$  kg<sup>-1</sup> for Type I and II (p < 0.05) respectively and the corresponding values for Na<sup>+</sup> were 102 and 98 (p < 0.05) mmol  $\times$  kg<sup>-1</sup>.

## Discussion

### *Separation of fibres in mixed muscles*

The method presented in this study offers a possibility to separate fibres in mixed muscles in order to perform quantitative biochemical determinations not only on muscles of a specific fibre type, but also on individual fibres. In addition to the technical difficulties in determining the wet weight and the ratio between extra- and intra-cellular volume of the fibres, the main limitation in the method lies in the evaluation of sensitive biochemical assays on freeze dried material.

Dissecting the fibres is a tedious procedure demanding skill and patience. It may take one hour to obtain 20-50 fibres. When the determinations are performed on single fibres it is of value to use as large fibres as possible. One important step is the classification of the fibres in Type I and II by staining for myofibrillar ATPase at pH 10.3 and 4.35. Although this proves difficult because a small number of fibres (<1-2%) do not stain either black or white, but more greyish. It is of note that when a single fibre has been cut in several pieces (5-10) and all pieces are stained for myofibrillar ATPase an identical stain has



Plate 3. Photomicrographs ( $\times 30$ ) illustrating 3 fragments from six different fibres placed in the order on three slides and stained for myofibrillar ATPase. In the left panel (slide) the preincubation was performed at a pH of 10.3, in the middle at pH 4.6 and to the right at pH 4.35. A type I fibre stains black (10.3), white (4.6) and black (4.35). The staining pattern for type II A fibre is black, white, white respectively at the three pH levels.



TABLE IV Comparison between quantitative biochemical measurements performed on different fibre types found in guinea pig (Peters *et al.* 1972), rat (Rustman *et al.* 1973), and muscles from man (Present study Table I). The ratio between Type II and I fibres (man) and between fast twitch glycolytic-oxidative (FTGO) and fast twitch glycolytic (FTG) and slow twitch oxidative (STO) fibres in guinea pig (rat) are given.

	Biochemical		
	Guinea pig/rat		Man
	FTGO/STO	FTG/STO	Type II/Type I
muscle characteristics			
young ATPase	2.4	2.3	2.8
actin			
myosin	2.9	2.2	1.0
myofibril	1.2	0.8	0.4
actin potential			
PK/z-glycophosphate			
hydrogenase	2.7	2.7	1.9
oxidative potential			
SDH	1.3	0.4	0.7

#### rat muscle

It is interesting to note our result on the SDH and PFK-activities of Type II (A and B) fibres. They do indicate definite differences between the two subgroups of Type II fibres. Whether a difference is genetic or due to an adaptation cannot be concluded at present. Preliminary studies using the disappearance of the PAS-stain from individual fibres have shown that at high work intensities demanding 80–90% of maximal oxygen uptake, Type II A fibres may lose PAS-stain at the same time as the Type I fibres (Andersen and Sjøgaard, personal communication). Type II B fibres in the same situation still stain intensely for glycogen. These results may then indicate different thresholds for activation of Type II A and B fibres, and the higher SDH-activity in the Type II A fibres is a consequence of more frequent use of these fibres than the Type II B. The conclusion must therefore be that human skeletal muscle does not contain a fast twitching fibre which has as high or higher oxidative capacity as the slow twitching fibres of the same muscle. A FTGO fibre with similar characteristics to the FTGO fibre in guinea pigs has been described in many different species including low forms of primates such as the Bush Babys (Gillespie, Simpson and Edgerton 1974). It would be of interest to investigate where in the phylogenetic scale the FTGO fibre becomes less oxidative than the ST-fibre.

The different fibre types of guinea pig muscles have different membrane potentials as well as potassium and sodium concentrations (Campion 1974). Also in human muscles different membrane potentials appear to exist between Type I and II fibres (Eberstein and Woodgold 1968, Hanson 1974), as well as contraction times (Buchthal and Schmalbruch 1970). We were unable to demonstrate significant differences in the K<sup>+</sup> and Na<sup>+</sup> concentration of Type I and II fibres. However we determined ion content on the whole fibre and a valid estimation of intra- versus extracellular concentration of these ions in the fibre must be made before a conclusion on this particular point can be drawn.

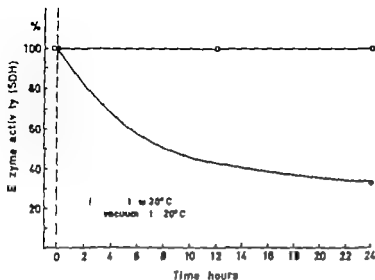


Fig. 3. The relationship between SDH activity and time in air at 20°C and in vacuum at 20°C.

#### *Muscle fibre types in man compared with other species*

The question arises as to whether skeletal muscles of man contain more than two distinctly different fibre types. A comparison by Peter *et al* (1972) of some of the biochemical characteristics quantitatively determined on "white" and "red" vastus and also on sole muscles of guinea pigs may be useful. The three muscles or portions of the muscles studied contain predominantly FT high glycolytic (FTG), FT high glycolytic-oxidative (FTGO) and ST high oxidative (STO) fibres (Gillespie, Simpson and Edgerton, 1970). The myosin ATPase appears to be very similar for human Type I and guinea pig STO fibres, whereas human Type II fibres have slightly higher ATPase activity than FTG and FTGO guinea pigs (Table IV). The glycogen content of human Type I fibres is considerably higher (5-6x) than that found in STO fibres of guinea pigs. In addition approximately 50% more glycogen is found in the human Type II fibres. This results, therefore, in FTG/STO or FTGO/STO ratios of 2-3 whereas Type II/I is 1.0 (Table IV). The lowest triglyceride content is found in human Type II and rat FTG. Thus the Type I fibre is well supplied both with glycogen and triglycerides. In fact skeletal muscle in man has 3-5 times more energy stored intracellularly than found in rats and guinea pigs.

No direct comparison between glycolytic enzymes can be made, but if the  $\alpha$ -glycerol phosphate dehydrogenase activity is used as an indicator of glycolytic potential in the guinea pig and PFK of that in human muscle fibres, fairly good agreement can be found although minor quantitative differences may exist in ratios (Table IV).

When considering the oxidative enzymes it is quite obvious that the human Type I (II A and B) and guinea pig FTG fibres exhibit the lowest activity whereas human Type II fibres are similar to the STO or FTGO fibres of guinea pigs. It may be argued that the very wide variation observed for both PFK and SDH activity in human Type I and II fibres should favour the idea of more than two fibre types. It must then be remembered that the measurements are performed on different functioning muscles of the body as well as on muscles that are in different states of training. In this connection it is

Table III Comparison between quantitative biochemical measurements performed on different fibre types found in guinea pig (Peters *et al.* 1972), rat (Riekman *et al.* 1973), and muscles from man (Present study Table I). The ratio between Type II and I fibres (mean) and between fast twitch glycolytic-oxidative (FTGO) and fast twitch glycolytic (FTG) and slow twitch oxidative (STO) fibres in guinea pig (rat) are given.

	Biochemical		
	Guinea pig/rat		Man
	FTGO/STO	FTG/STO	Type II/Type I
lactate characteristics			
total ATPase	2.4	2.3	2.8
creatine			
phospho	2.9	2.2	1.0
glyceral	1.2	0.5	0.4
phosphatase			
K, 2-glycerophosphate			
dehydrogenase	2.7	2.7	1.9
lactate phosphatase			
HH	1.3	0.4	0.7

in muscle.

Table III note our results on the SDH and PFK-activities of Type II (A and B) fibres. They do indicate definite differences between the two subgroups of Type II fibres. Whether a difference is genetical or due to an adaptation cannot be concluded at present. Previous studies using the disappearance of the PAS-stain from individual fibres have shown that at high work intensities demanding 80-90% of maximal oxygen uptake, Type A fibres may lose PAS-stain at the same time as the Type I fibres (Andersen and Sjøgaard, personal communication). Type II B fibres in the same situation still stain intensely for glycogen. These results may then indicate different thresholds for activation of Type II A and B fibres, and the higher SDH-activity in the Type II A fibres is a consequence of more frequent use of these fibres than the Type II B. The conclusion must therefore be that human skeletal muscle does not contain a fast twitching fibre which has as high or higher oxidative capacity as the slow twitching fibres of the same muscle. A FTGO fibre with similar characteristics to the FTGO fibre in guinea pigs has been described in many different species including low forms of primates such as the Bush Babys (Gillespie, Bapton and Edgerton 1974). It would be of interest to investigate where in the phylogenetic tree the FTGO fibre becomes less oxidative than the ST-fibre. The different fibre types of guinea pig muscles have different membrane potentials as well as potassium and sodium concentrations (Campion 1974). Also in human muscles different membrane potentials appear to exist between Type I and II fibres (Eberstein and Woodgold 1968, Hanson 1974), as well as contraction times (Buchthal and Schmalbruch 1970). We were unable to demonstrate significant differences in the K<sup>+</sup> and Na<sup>+</sup> concentrations of Type I and II fibres. However we determined ion content on the whole fibre and estimation of intra- versus extracellular concentration of these ions in the fibre must be made before a conclusion on this particular point can be drawn.

### *Interconversion of fibre types*

Experiments with electrical stimulation or cross-innervation in animals have shown that fibres can be converted one into the other (Bárány and Close 1971, Sjöström *et al.* 1970). Whether this occurs in human skeletal muscles is unknown. Different training procedures appear to have little influence on muscle fibre composition when based on the same myofibrillar ATPase (Gollnick *et al.* 1973, Saltin *et al.* 1975). However, this may not hold for the subgroups of Type II A and II B fibres as endurance training appears to increase the percentage of II A on the expense of II B fibres (Jansson 1975, Andersen and Henriksson, personal communication). In fact, a conversion of Type I to Type II fibres may occur in human skeletal muscle. Recently it has been found that patients who due to a lesion of the spinal cord are paretic in their lower extremities and develop spastically lined muscles to 100% Type II fibres in their thigh, gastrocnemius, and soleus muscles, whereas the deltoid muscles are mixed (Grumby, personal communication).

### *Muscle fibre nomenclature*

A long-standing controversy exists concerning the nomenclature to be employed for different muscle fibre types. Peter Barnard, Edgerton and colleagues (1972) have proposed a nomenclature for designating fibres which includes not only the fibres' contractile but also their metabolic characteristics. However, such a nomenclature is based on the assumption of a close correlation between enzyme activity levels of a fibre and the contractile characteristics as well as the metabolism taking place in it, which remains to be proven for human muscle. Moreover, the differences between human muscle fibre types may not be as distinct as in most other species. Although all this is true, it may still be worthwhile to designate human muscle fibres and fibre types by their basic characteristics in a manner similar to what has been done by Peter *et al.* (1972). Type I, II A and II B fibres would then be called slow twitch, high oxidative, low glycolytic, fast twitch, oxidative-glycolytic and fast twitch, low glycolytic, low oxidative fibres, respectively. In various species the absolute values for the contractile and metabolic characteristics may be of completely different magnitude. If the different terms when based on histochemical stains (slow-fast, low-high) are only qualitative and cannot be used for quantitative comparisons of muscle fibre types in different species. Finally it may be worthwhile to emphasize that from functional and clinical standpoints a subdivision of fibres beyond the two major groups of slow and fast twitch fibres may not so often be warranted.

This study was made possible by grants from the Swedish Medical Research Council (04X-4354) and Danish Natural Science Research Council.

### References

- BÁRÁNY, M. and R. J. CLOSE, The transformation of myosin in cross-innervated rat muscles. *J. Physiol. (Lond.)* 1971, 213, 435-474.
- BILGISTRÖM, J. Muscle electrolytes in man. *Scand. J. clin. Lab. Invest.* 1962, Suppl. 68.
- BROOKER, M. H. and K. K. KAISER, Muscle fibre types: How many and what kind? *Arch. of Neurol. (Chic.)* 1970, 23, 369-379.

- FRANK, F. and H. SCHMALBRUNN, Contraction time and fibre types in intact human skeletal muscle. *in physiol. scand.* 1970. 79. 435-452.
- FRANK, D. S., Resting membrane potential and ionic distribution in fast and slow-twitch mammalian muscle. *J. clin. Invest.* 1974. 54. 514-518.
- FRANK, D. S., Determination of glycerol and acylglycerol. *Methods in Enzymology* 1969. 14. 627-630.
- FRANK, A. and J. GOODGOLD, Slow and fast twitch fibres in human skeletal muscle. *Amer. J. Physiol.* 1968. 215. 545-547.
- FRANK, W. K., The necessity of histo- and cytochemical studies of skeletal muscle in investigation of neuromuscular diseases. *Neurology* 1962. 12. 778-794.
- FRANK, R. and J. HANAUER, Glycogen content of individual muscle fibres in man. *Acta physiol. scand.* 1974. 90. 645-647.
- FRANK, C. A., D. R. SAMPSON and V. R. EDGERTON, High glycogen content of red as opposed to white skeletal muscle fibres of guinea pigs. *J. Histochem. Cytochem.* 1970. 18. 552-558.
- FRANK, C. A., D. R. SAMPSON and V. R. EDGERTON, Motor unit recruitment as reflected by muscle fibre glycogen loss in *prosimia* (baboon) after running and jumping. *J. Neurol. Neurosurg. Psychiat.* 1974. 37. 817-824.
- FRANK, P. D., R. B. ARMSTRONG, C. W. SAUBERT IV, K. PETER and B. SALTIN, Enzyme activity and fiber composition in skeletal muscle of untrained and trained man. *J. appl. Physiol.* 1972. 33. 312-319.
- FRANK, P. D., R. B. ARMSTRONG, B. SALTIN, C. W. SAUBERT IV, W. L. SACHSOWITZ and R. E. SACHSOWITZ, Effect of training on enzyme activity and fiber composition of human skeletal muscle. *J. appl. Physiol.* 1973. 34. 107-111.
- FRANK, L. and F. J. SAMARA, Qualitative differences between acetylcholine ATPase of slow and fast mammalian muscle. *Exp. Neurol.* 1969. 25. 138-152.
- FRANK, L., Effects of repetitive stimulation of membrane potentials and twitch in human and rat intact skeletal muscle fibers. *Acta physiol. scand.* 1974. 92. 238-248.
- FRANK, E., Type II A and B fibres in skeletal muscle of man. *Acta physiol. scand.* 1975. 94. In press.
- FRANK, M. A., J. POLGAR, D. WICKSTROM, and D. APPLTON, Data on distribution of fibre types in activity in human muscles. An Autopsy Study. *J. Neurol. Sci.* 1972. 18. 111-129.
- FRANK, O. H. and L. V. PANDOLFI, *A flexible system of enzymatic analysis*. Acad. Press, New York, 1973.
- FRANK, O. H., M. J. ROBINSON, A. L. FARR and R. J. RANDALL, Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 1951. 193. 265-273.
- FRANK, H. A. and E. BERMAN, The specificity of the histochemical method of adenosine triphosphatase. *Histochem. Cytochem.* 1955. 3. 170-195.
- FRANK, J. B., R. J. BARNARD, V. R. EDGERTON, C. A. GALLISPE and K. E. STENGEL, Metabolic profiles of three fibre types of skeletal muscle in guinea pigs and rabbits. *Neuroscience* 1972. 11. 2627-2633.
- FRANK, J. E. M. BALDWIN and J. O. HOLLOMAN, Intramuscular triglyceride utilization by red, white and intermediate skeletal muscle and heart during submaximal exercise. *Proc. Soc. exp. Biol. (N.Y.)* 1973. 142. 422-431.
- FRANK, M. and P. W. HARRIS, Colorimetric determinations of inorganic phosphate in microgram quantities. *Anal. Chem.* 1951. 23. 1900-1901.
- FRANK, B. D., L. COSTILL, K. NAZAR, E. STENGEL, E. JAMNICK, B. BISH and H. D. GOLLIGOSKY, The nature of training response: Peripheral and general adaptations to one-legged exercise. *Acta physiol. scand.* 1973. In press.
- FRANK, P. A., J. GIBSON, S. SALMONS and P. C. A. ROMANUS, Synthesis by fast muscle of myosin light chain characteristics of slow muscle in response to long-term stimulation. *Nature New Biol.* 1973. 24. 17-19.
- FRANK, A. W. B. ENRI and B. SALTIN, Myosin ATPase in skeletal muscle of healthy man. *Acta physiol. scand.* 1974. 91. 548-570.

## The Postnatal Development of Some Twitch and Fatigue Properties of the Ankle Flexor and Extensor Muscles of the Cat

By

C HAMMARBERG and J-O KELLERTH

Received 27 March 1975

---

### Abstract

HAMMARBERG C and J-O KELLERTH. *The postnatal development of some twitch and fatigue properties of the ankle flexor and extensor muscles of the cat* Acta physiol. scand. 1975. 95. 166-178

The isometric responses of the medial gastrocnemius (MG), soleus (SOL) and anterior tibial (TA) muscles to single shocks and different modes of repetitive stimulation were studied in litters of various postnatal ages and in adult cats. The postnatal decrease in time-to-peak and half relaxation time of the twitches was similar for the MG and TA muscles and adult values were attained at around 6-7 weeks of age. The SOL muscle displayed a transient decrease in contraction time during the first postnatal week followed later by slowing towards adult values. The susceptibility to fatigue during iterative stimulation was smallest in the SOL at all ages studied, and usually largest in TA. It changed only little for the MG and SOL postnatally while increasing markedly for the TA up until 6-7 weeks of age. Tetanic contraction resulted in similar depressions in contractile tension of all three muscles in the youngest litters, the SOL displayed a greater ability to recover from this depression than the MG and, in particular, the TA muscles. Tetanus resistance increased postnatally and adult responses were attained at 6-7 weeks of age.

---

It has long been recognized (see Ranvier 1874) that the limb muscles of adult mammals may be classified into so-called "fast" and "slow" twitch muscles which differ in their speeds of contraction. As was first described by Banu (1922), however, at birth all the limb muscles of the kitten contract slowly, the twitch response having a time course similar to that of adult slow twitch muscles. The ensuing process of limb muscle differentiation during the postnatal stages of cat ontogeny has been the subject of several investigations dealing with, for example, isometric twitch characteristics (Denny Brown 1929, Buller *et al.* 1965), twitch/tetanus ratios (Buller and Lewis 1965, Close and Hoh 1967), force/velocity properties (Close and Hoh 1967) and post-tetanic potentiation of peak tetanic tension (Buller and Lewis 1965, Nyström 1968a, f). An additional important aspect of muscle performance which has received less attention is the sensitivity to fatigue during prolonged activity. In the adult cat Burke *et al.* (1971, 1973, 1974) found this parameter to be of major significance in the classification of different types of motor units. The present investigation was undertaken to study the changes in some twitch and fati-

peries which occur in the anterior tibial, medial gastrocnemius and soleus muscles of cat during postnatal development. The results obtained also serve as a basis for following biological studies on the postnatal differentiation of various motor unit types in the skeletal muscles (Hammarberg and Keilerth 1975 b). The histochemical staining properties of these muscles during postnatal and adult stages have been described previously (Hammarberg 1974 a, b).

## Methods

experiments were performed on 7 adult cats and 16 kittens varying in age between 6 and 65 days. Animals were anaesthetized with 40 mg/kg of pentobarbitone sodium (Nembutal) given intraperitoneally. Subsequent doses, when necessary, were given through catheter inserted in the left femoral vein. Animals were usually allowed to breathe spontaneously through tracheal cannula, and expired  $\text{CO}_2$  monitored continuously. In one adult animal artificial respiration was employed in order to study the effects of hyper- and hypoventilation upon muscle performance (see Fig. 1 and 2).

In anterior tibial (TA), medial gastrocnemius (MG) and soleus (SOL) muscles of the right hind limb exposed and dissected free except for their proximal attachments, care being taken to preserve their blood and nerve supplies. The corresponding motor nerves were cut in the popliteal fossa and mounted on silver electrodes. In some animals muscles from both legs were used for the experiment. The animals were mounted in steel frames with the long axis of the hind limb almost horizontal. A steel drill transmitted distal end of the femur while the distal ends of the tibia and fibula were rigidly clamped. The cut distal ends were arranged for separate isometric connections to strain gauge myograph through lengths of braided suture (compliance 0.00 mm/kg cm). Care was taken to align the strain gauge with the natural direction of muscle pull. The strain gauge was attached to sliding metal rod provided with each leg made possible to attend the muscles continuously by micrometer. In order to provide standard by which to compare the contractions of the different muscles at various postnatal ages, the origin of each muscle was always adjusted to give maximal twitch responses (Baker *et al.* 1969). By elevating the surrounding skin flaps it was possible to immerse the exposed tissues in pool of paraffin oil. Muscle temperature as measured by means of infrared light. With the youngest kittens, however, the small volumes of the paraffin oil pools made control difficult and variations in intramuscular temperature between 34–38°C had to be accepted. Body temperature was kept at 36–38°C.

Muscle contractions were evoked by stimulating the appropriate motor nerves with supra-maximal square wave pulses of 0.5 ms duration. Because of the varying ages of the animals strain gauges of different maximal strains had to be used to record the mechanical responses. The unloaded natural resonant frequency of the strain gauges exceeded 350 Hz in all cases. The output of the strain gauge was amplified and displayed on oscilloscope and on penwriter (Devices M3). The oscilloscope traces were photographed on paper.

## Measuring procedures

1. **Twitch contractions** were elicited by single stimulating pulses. The "twitch rise time" was measured from the start of the contraction to its peak, while the "twitch half-relaxation time" was measured from the peak to the point representing 50% passive decay in twitch tension. Because the speed of muscle contraction increases with temperature coefficient and because muscle temperature was often difficult to control in smaller kittens, the recorded values for contraction time have been adjusted in Fig. 3 to give the corresponding values at 20°C by using the correction factor  $T_{0.5} = 1.55$  (Gordon and Phillips 1953, Baker *et al.* 1969). The maximal adjustment made in Fig. 3 amounted to 21% reduction of the recorded values.

2. **Fatigue properties** of the muscles were examined by the following series of tests:

1. **Fatigue test** Short trains of stimuli with stimulus frequency of 40 Hz and duration of 330 ms were repeated once a second (see Fig. 1 and 2). This test, which will be referred to below as the "fatigue test" was originally introduced by Burke *et al.* (1971) to assess the sensitivity to fatigue of single motor units. The amount of fatigue exhibited by the various muscles after ten minutes of stimulation will be expressed as the "fatigue index" i.e. the ratio of the tension output during the 120th tetanus to the tension produced by the first tetanus of the sequence.

2. **Pauses test** After about 2 min of the "fatigue test" stimulation was suddenly interrupted for ten

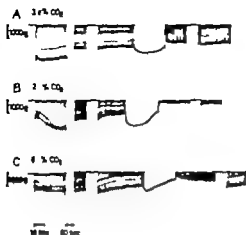


Fig. 1

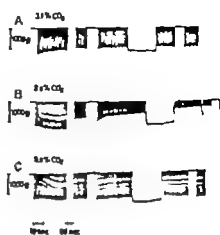


Fig. 2

Fig. 1 and Fig. 2. The contractile responses of the MG (Fig. 1) and SOL (Fig. 2) muscles of 22 g during the "fatigue", "tetanus" and "pause tests". In each record the concentration of expired  $\text{CO}_2$  has been indicated. *A*: Normal breathing. *B*: Hyperventilation. *C*: Hypoventilation. It is seen that in either direction from normal ventilation  $\text{CO}_2$  markedly affects the stability of both fast and slow components to maintain their contractile tensions during sustained activity. *B* and *C* were recorded after 5 min of normal and hypoventilation, respectively, when the new concentration of expired  $\text{CO}_2$  had reached its steady state. Between *B* and *C* the animal was allowed to breathe spontaneously for 45 min which was sufficient for the complete restitution of normal contractile responses seen in *A*.

seconds. This is referred to as the "pause test". When stimulation was resumed the amount of recovery in contractile tension following the short rest was studied (see Fig. 1 and 2).

3. "Tetanus test". Approximately 30 s after the "pause test" when tetanic output had returned to or less steady level the muscle was exposed for 30 s to sustained tetanic stimulation at 100 Hz (see Fig. 1 and 2). The iterative stimulation was then resumed and the changes in tetanic output or both during and after this so-called "tetanus test" were studied.

4. About 30–45 s after the end of the maintained tetanic stimulation a second "pause test" was performed and the ensuing recovery in contractile tension measured.

## Results

**Twitch rise times** (time-to-peak) of the MG, SOL and TA muscles at various postnatal ages are shown in Fig. 3. The results are essentially in agreement with the observations of Buller *et al.* (1960), Buller and Lewis (1965), Mann and Salafsky (1970) and West *et al.* (1973). In the smallest kittens all three muscles displayed similarly slow contractile times, although there was a tendency for the SOL muscle to have a somewhat higher mean rise time in all the age groups studied here. There was a parallel decrease in rise time for the three muscles during the first 3–4 weeks, after which the SOL rise time increased while the rise times of the other two muscles continued to decrease. It appears, therefore, that the soleus contracts faster at around 3 weeks of age than in the adult stage ( $p < 0.002$ ). The striking difference between the adult and kitten twitches is seen in the fast MG and TA muscles, the adult muscles being markedly faster. The adult values for twitch rise times were fairly well acquired by the fast muscles at around 6–7 weeks of age, while those of the SOL muscle were acquired somewhat later (cf. also Buller *et al.* 1960, and Mann and Salafsky 1970). In all age groups studied here there was a consistent tendency for the TA to contract slightly faster than the MG.



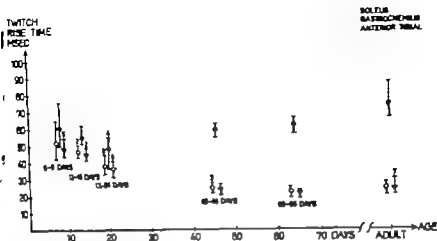


Fig. 3. The postnatal changes in twitch rise time (time-to-peak) for the MG, SOL and TA muscles. Each bar shows the range of observed values, the number of each is indicated above each bar. The round and square symbols show the mean values.

**Twitch half-relaxation time (HRT)** Fig. 4 illustrates the postnatally occurring changes in HRT of the three muscles. Here also the fast MG and TA muscles acquired their adult form at around 6-7 weeks of age, while the SOL possibly took somewhat longer. The mean values for HRT at different ages are generally consistent with the findings of Butler *et al.* (1960) and Mano and Salafsky (1970) except in the case of kittens around 1 week of age, where the present study found the value for SOL in particular to be considerably higher than in the earlier studies (HRT mean values  $48.0 \pm 15.5$  ms and  $35.5 \pm 9.9$  ms for

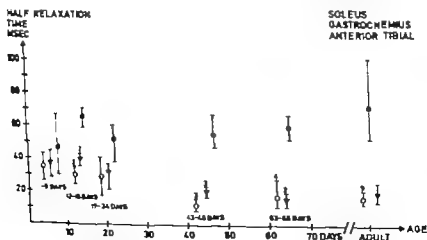


Fig. 4. The postnatal changes in twitch half-relaxation time for the MG, SOL and TA muscles. Each bar indicates the range of observed values, while the round and triangular symbols show the mean values. The number of observations is indicated above each bar.

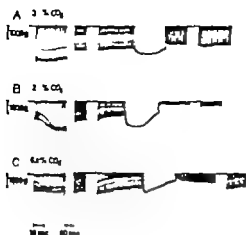


Fig. 1

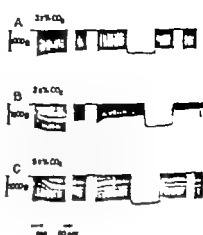


Fig. 2

Fig. 1 and Fig. 2. The contractile responses of the MG (Fig. 1) and SOL (Fig. 2) muscles of adult during the "fatigue" tetanus and "pause" tests. In each record the concentration of expired  $\text{CO}_2$  has been indicated. A: Normal breathing. B: Hyperventilation. C: Hypoventilation. It is seen that deviation in either direction from normal ventilation will markedly affect the ability of both fast and slow muscles to maintain their contractile tensions during sustained activity. B and C were recorded after 5 min of hyperventilation and hypoventilation, respectively when the new concentration of expired  $\text{CO}_2$  had reached its steady state. Between B and C the animal was allowed to breathe spontaneously for 45 min which was sufficient for the complete resumption of normal contractile responses seen in A.

seconds. This is referred to as the "pause test". When stimulation was resumed the amount of residual contractile tension following the short rest was studied (see Fig. 1 and 2).

3. "Tetanus test". Approximately 30 s after the "pause test" when tension output had returned to or less steady level, the muscle was exposed for 30 s to sustained tetanic stimulation at a frequency of 40 Hz (see Fig. 1 and 2). The literature stimulation was then resumed and the changes in tension output occurred both during and after this so-called "tetanus test" were studied.

4. About 30–45 s after the end of the maintained tetanic activation a second "pause test" was performed to study the ensuing recovery in contractile tension measured.

## Results

**Twitch rise times** (time-to-peak) of the MG, SOL and TA muscles at various postnatal ages are shown in Fig. 3. The results are essentially in agreement with the observations of Bulter *et al.* (1960), Bulter and Lewis (1965), Mann and Salafsky (1970) and Westberg *et al.* (1973). In the smallest kittens all three muscles displayed similarly slow contractions, although there was a tendency for the SOL muscle to have a somewhat higher mean value. In all the age groups studied here. There was a parallel decrease in rise time for the 3 muscles during the first 3–4 weeks, after which the SOL rise time increased, while the rise times of the other 2 muscles continued to decrease. It appears, therefore, that the soleus muscle contracts faster at around 3 weeks of age than in the adult stage ( $p < 0.002$ ). The most striking difference between the adult and kitten twitches is seen in the fast MG and TA muscles, the adult muscles being markedly faster. The adult values for twitch rise time were fairly well acquired by the fast muscles at around 6–7 weeks of age, while those of the SOL muscle were acquired somewhat later (cf also Bulter *et al.* 1960, and Mann and Salafsky 1970). In all age groups studied here there was a consistent tendency for the TA to contract slightly faster than the MG.

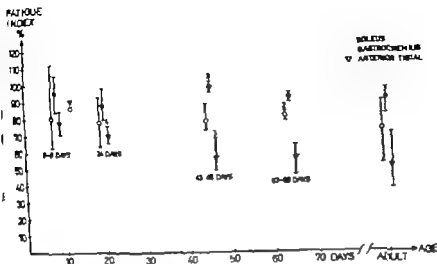


Fig. 6. The postnatal changes in "fatigue index" for the MG, SOL and TA muscles. Each bar illustrates the range of observed values, while the round and triangular symbols show the mean values. The number of observations is indicated above each bar.

tion of stimulation, although earlier in the sequence an initial increase reminiscent of post-tetanic potentiation had gradually disappeared.

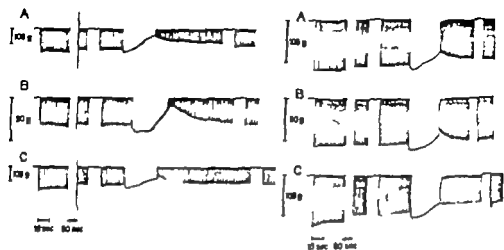
In the 18-day-old kitten a qualitatively similar "fatigue test" picture was obtained both in the MG and, in particular, the TA showed a progressive reduction in tension output during a 2 min. of stimulation, while the SOL remained virtually unaffected. The same general tendency was present in the 48-day-old kitten, as well as in the adult animal.

Fig. 6 illustrates the "fatigue indices" (see Methods) for each of the tested muscles at various postnatal ages. The change in fatigue index occurring during postnatal development is quite conspicuous in the case of the TA muscle which became less resistant to fatigue with increasing age ( $p < 0.01$ ). In the MG and SOL muscles, however, the changes were insignificant, and the relative difference in mean values between the two muscles remained almost constant across the age groups investigated. In the adult animal a clear difference in fatigue index was found between the TA and SOL muscles ( $p < 0.001$ ), while MG appeared to occupy a more intermediate position.

**Tetanus test.** Although the "fatigue indices" (Fig. 6) suggest that both the SOL and MG muscles maintain a more or less unchanged resistance to fatigue throughout the postnatal period, a different picture is obtained when the "tetanus test" is used. As seen in Fig. 5, all three muscles of the 7-day-old kitten showed considerable reduction in tension during the course of the 30 s long tetanic stimulation. Generally in this age group, the three muscles tested showed similar amounts of reduction in tension. At 18 days of age the ability to withstand the tetanus had improved, but no obvious differences were yet observed among the individual muscles. At around six to seven weeks of age, however, a pattern had emerged which strongly resembled that of the adult animal. In the SOL muscle the tetanic tension was

7 DAYS

18 DAYS



48 DAYS

ADULT

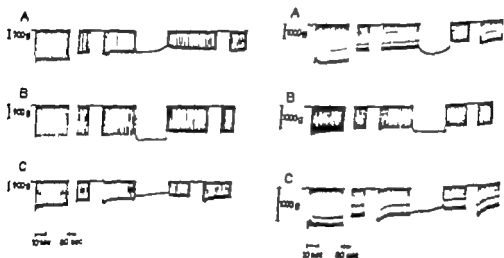


Fig. 5 Typical examples of the contractile responses of the MG (A) SOL (B) and TA (C) muscles to the fatigue tetanus and pause tests at different postnatal ages.

SOL and MG respectively compared to approximately 70–75 ms and 40–45 ms, respectively according to Fig. 4 of Buller *et al.* 1960). With respect to the general pattern of motion the contraction times (Fig. 3) and half-relaxation times (Fig. 4) for the different muscles were found to display similar time courses (see also Buller *et al.* 1960).

“Fatigue test” Fig. 5 shows some typical examples of the behaviour of the MG, SOL and TA muscles (A, B and C respectively) at different postnatal ages during prior activity.

The MG and TA muscles (A and C, respectively) of the 7-day-old kitten of Fig. 5 show a moderate reduction in contractile tension after 2 min of intermittent stimulation, reduction being somewhat more pronounced for the latter muscle. The SOL muscle on the other hand, produced approximately the same amount of contractile tension:

as it has been shown that such differences are reflected in the size of their vascular supplies (Romanul 1965). Alternatively these effects might also be caused by extracellular accumulation of potassium or metabolites in the muscles (Littigau 1965).

The "post-tetanic period" which followed the 30 s tetanus, also deserves comment. In the 7-day-old kitten (see Fig. 5) the tetanus always induced a considerable initial reduction in the amplitude of the following repetitive contractions. This reduction usually was of similar magnitude for the 3 muscles tested. It did not persist, however, but a gradual recovery in contractile tension started almost immediately and proceeded to a certain plateau. Although the total amount of recovery achieved by a particular muscle varied somewhat between animals, it was consistently found to be largest in the SOL muscle and usually smallest in the TA muscle. In the 2- to 3-week-old kittens the initial depression in contractile strength induced by the tetanus had decreased in all three muscles, but the strong tendency for recovery in the SOL muscle was still evident. The TA muscle generally had a poorly developed recovery ability at this age, while the MG showed a behaviour intermediate to the other two muscles. In the 6- to 7-week-old kittens and adult animals the initial depression in contractile strength following the tetanus was either absent or very small, indicating that the muscles reached their post-tetanic plateaus quickly. Typically in these age groups post-tetanic SOL contractions had approximately the same amplitude as contractions before the tetanus, and TA contractions were generally somewhat more depressed post-tetanically than the MG ones (cf. Fig. 5).

It appears, therefore, that with regard to the changes in contraction strength which occur during the "post-tetanic period" a difference between the muscles is already present at 7 days of age. The SOL muscle exhibited a larger post-tetanic recovery than the other muscles, while the TA contractions were usually more depressed than those of the MG. Qualitatively the same tendencies remained throughout the different age groups studied.

The pause test was applied to each muscle both before and after the "tetanus test". The intention was to study the amount of recovery in contractile tension immediately after a rest. In general, the second of these "pause tests" was found to be more sensitive than the first in displaying possible differences in recovery properties between the various muscles (cf. Fig. 5). This would be expected since particularly in the youngest kittens the muscles appeared to be less exhausted after 2 min of iterative stimulation than after the later "tetanus test" (see Fig. 5). Fig. 8 illustrates for each muscle at various postnatal ages the relative amount of recovery in contractile tension following the second "pause test" which was initiated about 30-45 s after the end of the tetanus. Although a considerable spread of values was found for muscles of the same postnatal age the mean values ( $\pm 3$  E.) for recovery were almost the same as one week postnatally (MG  $15.3 \pm 6.2\%$ , TA  $23.4 \pm 13.0\%$ , SOL  $5.4 \pm 0.6\%$ ) as at the adult stage (MG  $14.5 \pm 8.4\%$ , TA  $28.7 \pm 9.0\%$ , SOL  $6.5 \pm 6.0\%$ ). An important finding which is not evident from Fig. 8 is that in all the animals studied there was only one exception (the 7-day-old kitten of Fig. 5) to the general rule that the TA muscle always exhibited the largest amount of recovery in contractile tension after the pause, while the SOL muscle always showed recovery which was less than that of MG. This increase in TA contractile tension after the pause was usually short-lasting, however, and disappeared gradually during the following 10-20 s of iterative stimulation.

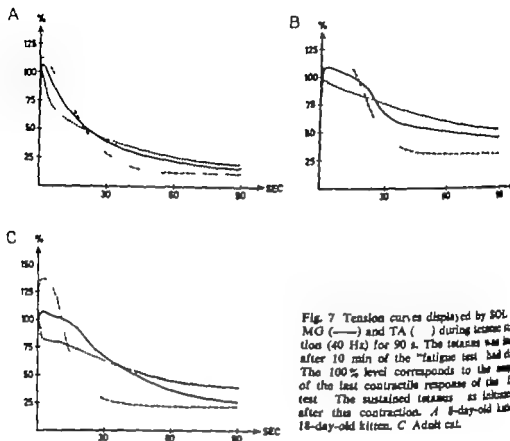


Fig. 7 Tension curves displayed by SOL, MG (—) and TA (---) during tetanic stimulation (40 Hz) for 90 s. The tetanus was initiated after 10 min of the "fatigue test" had elapsed. The 100% level corresponds to the amplitude of the last contractile response of the test. The sustained tetanus as indicated after this contraction. A 8-day-old kitten. B 18-day-old kitten. C Adult cat.

well maintained throughout the period of stimulation and, furthermore, its magnitude always exceeded the tension produced by the intermittent stimulation by 10%–40%. In the SOL muscle the tetanic tension declined continuously throughout the stimulation period and the initial peak amplitude never exceeded the contractile tension produced by the preceding intermittent activation. The MG muscle usually showed a gradual increase in tension during the first 10–15 s, followed by a smooth decline. In the adult animals the peak of this rather complex tension curve always represented a tension exceeding that produced by the intermittent stimulation immediately prior to the tetanus.

In a few animals the continuous tetanic stimulation was not started until 10 min after a "fatigue test" had elapsed. It was then maintained for 90 s. As seen in Fig. 7 the SOL muscle at all ages was more depressed by this kind of stimulation than were the other two muscles, although the difference between the muscles was rather small in the youngest kittens. These results may appear to be in disagreement with those of the "tetanus test" (see above) but particularly in the older animals, the SOL was always found to be more fatigue resistant than the MG and TA muscles (see Fig. 5). However, since the pattern of fatigue illustrated in Fig. 7 was never observed when studying single motor units of different types (Hammarberg and Kellerth 1975 a, b) these results may be interpreted as being due to local effects caused by occlusion of the blood supply of the muscles resulting from the prolonged tetanic contraction (cf Emmelin and MacIntosh 1956). The fatigue curves of Fig. 7 would then rather be taken to display differences in aerobic metabolism among the muscles.

ported by Buller *et al.* (1960), possibly due to small temperature differences (Gordon and Lips 1953, Buller *et al.* 1968).

A gradual decrease in contraction time of the SOL was observed during the first 3 postnatal weeks, followed by an increase approaching adult values which were achieved later on in the case of the fast muscles (see also Buller *et al.* 1960, Mann and Salafsky 1970). It is interesting to note, in this connection, that histochemical studies have demonstrated variation in staining patterns among fibres of the MG, TA and SOL muscles up until 3-4 weeks postnatally after which age SOL alone gradually loses its differentiated character and attains its adult homogeneous appearance by 7-10 weeks postnatally (Karpati and Engel 1967, Nystrom 1968 g, Hammarberg 1974 b).

The pattern of postnatal changes in HRT is generally consistent with that described by various authors (Buller *et al.* 1960, Mann and Salafsky 1970) except in the case of the youngest kittens, where the soleus HRT in particular was found to be shorter than expected. The gradual decrease in soleus HRT which has been reported by others to occur during the first 3 postnatal weeks (e.g. Buller *et al.* 1960) was therefore not substantiated in the present study nor in a subsequent investigation of single motor units (Hammarberg and Kellerth 1975 b). We cannot at this point, however, offer any satisfactory explanation for the discrepancy between these results. The HRT of the MG and TA was found to decrease directly with the contraction time, the adult values being attained at around 6-7 weeks postnatally.

The 'fatigue indices' observed in adult animals suggest that differences exist among the mature muscles in terms of their sensitivity to fatigue, SOL being least susceptible to fatigue and TA most susceptible. These findings probably reflect differences in the mature muscles with regard to their relative composition by various functional types of motor units (Burke *et al.* 1973, 1974, Proke and Wase 1974, Hammarberg and Kellerth 1975 a). The relatively homogeneous composition of SOL with respect to muscle fibre physiology and histochemistry has been verified repeatedly (see e.g. Henneman and Olsson 1965, Nystrom 1968 g, Burke *et al.* 1974, Hammarberg 1974 a). It would also appear reasonable that the MG as an antigravity muscle would contain a greater proportion of fatigue resistant units than the TA, which is engaged more in phasic movements with little load. In the youngest kittens the three muscles exhibited similar 'fatigue indices' as would be expected since muscle fibres are less differentiated at this age (Banu 1922, Denny Brown 1929, Buller *et al.* 1960, Nystrom 1968 g, Hammarberg 1974 b). The TA muscle showed a marked increase in sensitivity to fatigue with increasing age, adult values being attained at around 7 weeks postnatally. The MG and SOL muscles, on the other hand, maintained more or less unchanged 'fatigue indices' throughout the developmental period.

It should be pointed out, however, that with prolonged stimulation changes in amplitude of contractile responses are probably determined by two factors with opposite effects, namely potentiation and fatigue (on Euler and Swank 1940). In the smallest kittens potentiation following low-frequency tetanization has been found to be more prominent in the SOL than in the MG, while the reverse situation was found in adult animals (Standaert 1964, Nystrom 1968 a, f see also Burke *et al.* 1973, 1974). If the possible contribution of potentiation is taken into account, one cannot exclude the possibility that the differences

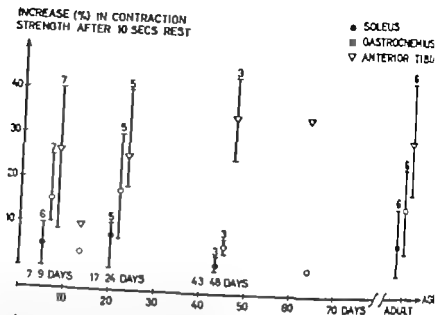


Fig. 8 The relative increase in contractile strength occurring in the MG SOL and TA muscles of the second pause-test plotted against postnatal age. The O-ordinate corresponds to the value of the last contraction prior to the pause. Each bar illustrates the range of observed values, while the symbols show the mean values. The number of observations is indicated above each symbol.

These findings suggest in summary that with regard to the pattern of recoverable contractile tension following the "tetanus test" differences already exist between the fast and slow hind limb muscles after the first postnatal week. The SOL muscle has a marked ability to overcome post tetanic depression in contractile tension, and this was usually more or less complete before the "pause test". The fast muscles, and particularly the TA, remained significantly more depressed post tetanically and even when by a 10 s rest only temporarily and to a limited degree approached the pre-tetanic contractile tension. The possible mechanisms underlying this early difference between the muscles will be discussed below.

### Discussion

The present study has been confined to an investigation of the changes in some basic fatigue properties which occur in the MG SOL and TA muscles of the cat during the postnatal period. The developmental changes in contraction time have previously been reported by several other investigators (Banu 1922, Denny Brown 1929, Buller *et al.* 1960, and Lewis 1965, Mann and Salafsky 1970, Westerman *et al.* 1973), and were included only for comparison with the pattern of changes in fatigue properties observed in the adult animals.

The postnatal changes in contraction time of the potentially fast MG and TA muscles were found to follow a time course similar to that reported by previous authors (*e.g.* Buller *et al.* 1960, Buller and Lewis 1965) and adult values were recorded at 6-7 weeks of age. The contractions were generally somewhat faster particularly in the youngest kittens, than in



parison of the flexor motor system (Kellerth *et al.* 1971; Mellstrom 1971; Hammarberg 1971) also appear incompatible with the view that this system would be more apt to fail synaptic spike propagation or in neuromuscular transmission than would the extensor system. In our opinion, therefore, it seems more likely that the observed differences in the properties of the immature muscles will be found postsynaptically where differences in post-tetanic potentiation have been shown to exist (Nyström 1968f).

This study was supported by grants from the Swedish Medical Research Council (project No 2444) and the Swedish Research Council for Muscular Diseases.

## References

- ALLAN, J. D. (1971). *Recherches sur le développement neuromusculaire*. Paris 1971.
- ALLAN, J. D., J. C. ECCLES and R. M. ECCLES. Differentiation of fast and slow muscles in the cat hind limb. *J. Physiol. (Lond.)* 1960 130 399-416.
- ALLAN, J. D. and D. M. LEWIS. The rate of tension development in isometric tetanic contractions of mammalian fast and slow skeletal muscles. *J. Physiol. (Lond.)* 1965 176 337-354.
- ALLAN, J. D., K. W. RAMANUNJAN and J. M. SARTS. The influence of temperature on the contractile characteristics of mammalian fast and slow twitch skeletal muscles. *J. Physiol. (Lond.)* 1968 196 81P.
- ALLAN, J. D., D. N. LEVINE, F. E. ZAJAC, III, P. TRACER and W. K. ENGEL. Mammalian motor units: Physiological-histochemical correlation in three types in cat gastrocnemius. *Science* 1971 174 709-714.
- ALLAN, J. D., D. N. LEVINE, P. TRACER and F. E. ZAJAC, III. Physiological types and histochemical profiles of motor units of the cat gastrocnemius. *J. Physiol. (Lond.)* 1973 231 723-748.
- ALLAN, J. D., D. N. LEVINE, M. SALOMON and P. TRACER. Motor units in cat soleus muscle: Physiological, histochemical and morphological characteristics. *J. Physiol. (Lond.)* 1974 238 503-514.
- ALLAN, J. D. and J. F. Y. HOE. Force-velocity properties of kitten muscles. *J. Physiol. (Lond.)* 1967 192 13-22.
- ALLAN, J. D. The histological features of striped muscle in relation to its functional activity. *Proc. Roy. Soc. B* 1929 104 371-411.
- ALLAN, J. D. The blockage of motor impulses in an asynchronous volley at the neuromuscular junction. *Comp. Physiol.* 1953 46 348-350.
- ALLAN, J. D. and F. C. MACINTOSH. The release of acetylcholine from perfused sympathetic ganglia and skeletal muscles. *J. Physiol. (Lond.)* 1956 131 477-496.
- ALLAN, J. D. and R. L. SEWELL. Tension changes during tetanus in mammalian and avian muscle. *J. Physiol. (Lond.)* 1940 9 203-219.
- ALLAN, J. D. P. Studies on the neuromuscular junction. IV. The nature of posttetanic inhibition. *Can. J. Physiol.* 1937 11 437-450.
- ALLAN, J. D. and C. G. PHILLIPS. Slow and rapid components in skeletal muscle. *Quart. J. exp. Physiol.* 1933 28 5-15.
- ALLAN, J. D. Histochemical staining patterns of muscle fibres in the gastrocnemius, soleus and anterior tibial muscles of the adult cat, as viewed in serial sections stained for lipids and succinate dehydrogenase. *Acta Anat. Scandinavica* 1974 90 272-284.
- ALLAN, J. D. The histochemical appearance of developing muscle fibres in the gastrocnemius, soleus and anterior tibial muscles of the kitten, as viewed in serial sections stained for lipids and succinate dehydrogenase. *Acta Anat. Scandinavica* 1974b 90 283-301.
- ALLAN, J. D., C. and J. O. KELLERTH. Studies of some twitch and fatigue properties of different motor unit types in the soleus muscles of the adult cat. *Acta physiol. scand.* 1975a 95 231-242.
- ALLAN, J. D. and J. O. KELLERTH. The postnatal development of some twitch and fatigue properties of single motor units in the soleus muscles of the kitten. *Acta physiol. scand.* 1975b 95 243-257.
- ALLAN, J. D. and C. B. OLSON. Relations between structure and function in the design of skeletal muscles. *Am. J. Anat.* 1963 26 381-392.
- ALLAN, J. D. and W. K. ENGEL. Neuronal trophic function. *Arch. Neural.* 1967 17 542-543.
- ALLAN, J. D., A. M. LESTROM and S. SUNDLUND. Postnatal excitability changes of kitten motoneurons. *Acta physiol. scand.* 1971 83 31-41.

in "true" fatigue between the muscles may be smaller in the youngest kittens and in the adults than the "fatigue indices" of the present study suggest. Such a possibility is supported by the results of the "tetanus test" where the intention was to produce fatigue in the immature muscles in order to compensate for any simultaneous potentiation effects. In this situation, all three muscles of the youngest kittens were found to respond in a similar way and the postnatal changes in their tetanus responses also followed a similar time course, the adult pattern being attained at around 6-7 weeks postnatally (see Fig. 4). The potentiation which would be expected to occur as a consequence of the prolonged stimulation during this "tetanus test" (Nyström 1968 a, f) may also help explain the differences in contractile behaviour of the immature muscles during the "post-tetanic potentiation". In the youngest kittens the SOL muscle showed a marked tendency to recover from its tetanically depressed state of tension output possibly due in part to strong FFP at this particular age (Nyström 1968 a, f). With increasing age the SOL gradually improved resistance to fatigue despite the simultaneous disappearance of potentiation (Nyström 1968 a, f). The fast muscles, on the other hand, remained quite depressed post-tetanic in the smallest kittens, due possibly to the lack of potentiation at this age (Nyström 1968 a, f). However even when supported by potentiation these muscles in the adult animal could not match the fatigue resistance of the SOL (Standaert 1964 Nyström 1968 a, f, see also Burke *et al* 1973 1974).

The term "fatigue" has been used in a rather unspecific sense in the present study to denote merely the decrease in contractile tension which occurs after prolonged stimulation. Obviously deficient contractions may result from failure of any one of several mechanisms and the type of failure may even be different in immature kittens and adult cats. The possibility of failure of initiation or propagation of impulses in the nerve stem fibres appears unlikely however since stimulus strength was always kept supramaximal. Furthermore when the same stimulus frequencies were used in studies on single motor units, a 1:1 input-output relationship was always found to exist between stimuli and EMG responses in both kittens and adult cats, even during fatigue (Burke *et al* 1973 1974 Hammarberg & Kellerth 1975 b). However the present results cannot exclude the possibility of a partial presynaptic failure of propagation involving more distal parts of an axon, such as one of its fine nerve terminals (Ramsay and Street 1942, Dun 1955 Krnjević and Miledi 1958 a failure of neuromuscular transmission (Feng 1937 Rosenbluth and Morison 1948 Krnjević and Miledi 1958 Shamulina 1961) or a postsynaptic failure of electrical coupling between contractile mechanisms in the muscles (Merton 1954, Krnjević and Miledi 1958, Lott 1965). There exists some evidence, however that the observed differences in fatigue properties between the potentially fast and slow muscles of the youngest kittens (see Fig. 4 and 8) may not be accounted for by corresponding differences in presynaptic mechanisms or in neuromuscular transmission. During the first 3-4 postnatal weeks the SOL and the fast muscles have previously been found to appear quite similar with respect to a number of parameters such as fibre diameter distribution and conduction velocity of the efferent nerve fibres, the relationship between muscle fibre diameters and size of nerve terminals, morphology of the motor end plates, ACh-esterase activity and increase in transmitter mobilization following conditioning tetanization (Nyström 1968 b-e). Furthermore, previous studies of an early

## The Influence of Rheological and Collapse Factors on Pre- and Post-Capillary Flow Resistances in the Skeletal Muscle Vascular Bed of the Cat

By

BENOT ÖBERG, ROD LITTLE, BENOT RIPPÉ and BJÖRN FOLKOW

Received 2 April 1975

### Abstract

ÖBERG, B., LITTLE, R., RIPPÉ, B. and FOLKOW, B. *The influence of rheological and collapse factors on pre- and postcapillary flow resistances in the skeletal muscle vascular bed of the cat.* Acta physiol. scand. 1975. 95: 179-190.

Importance of pressure-elastoc changes of vascular dimensions and shifts in regional effective viscosity on pre- and postcapillary resistance ratio ( $R_A/R_V$ ), and hence mean capillary pressure ( $P_{CB}$ ) was analysed in cat muscle. Reductions of mean venous distending pressure below 6-8 mm Hg induced marked  $R_A$  rises due to escalating venous collapse. This mechanism tends to deflect the  $P$  reductions and rate transcapillary fluid absorption during intense precapillary vasoconstriction. Comparisons of  $R_A/R_V$  erythrocyte suspensions (Hct 40-50) and cellfree perfusates at identical vascular dimensions showed  $R_A/R_V$  as considerably higher and  $P$  correspondingly lower for the erythrocyte suspension except at very low flows. This  $R_A/R_V$  difference increased with increasing flow and at very high flows  $P$  was at 10 mm Hg lower during perfusion with the erythrocyte suspension. These findings apparently diverge in the known influence of tube radius and linear flow velocity on effective  $\eta$  - i.e. viscosity of blood. In distal precapillary and proximal capillary sections, both having smaller diameters than the erythrocytes located upstream to the point of filtration-absorption equilibrium, they contribute in this respect to  $R_A$ . It is therefore suggested that the increasing  $R_A/R_V$  with erythrocyte perfusion, particularly at higher flows, is not due to genuine viscosity factors but to friction losses when cells in "bore flow" are squeezed with the narrowed precapillary sections.

The resistance to blood flow in the vascular bed is, according to the Poiseuille relation, determined by the vessel dimensions and the effective viscosity of the blood. Several studies (e.g. Whittaker and Winton 1933; Djojosingito *et al.* 1970), where blood viscosity  $\eta$  was determined at varying flow rates, indicate that effective blood viscosity is surprisingly low considering the high content of suspended red corpuscles. Thus, in the maximally dilated muscle vascular bed Djojosingito *et al.* (1970) observed an effective viscosity of 2.3-2.5 cP at high blood flows; that is, only about twice the value for plasma viscosity. Furthermore, as  $\eta$  blood viscosity increases surprisingly little even when flow is markedly reduced by vasoconstriction, then rarely exceeding 3 cP, i.e. only 30% higher

- KARZEVIC, K. and R. MILADY, Failure of neuromuscular propagation in rats. *J. Physiol. (Lond.)* 1960 440-461.
- LÖTTGAU H C., The effect of metabolic inhibitors on the fatigue of the action potential in single fibres. *J. Physiol. (Lond.)* 1963. 172. 45-67
- MANN, W. S. and B. SALATSKY, Enzymic and physiological studies on normal and denervated developing and slow cat muscles. *J. Physiol. (Lond.)* 1970. 208. 33-47
- MELLSTRÖM, A., Postnatal excitability changes of the ankle monosynaptic reflexes in the cat. *Acta physiol. scand.* 1971. 82. 477-489
- MERTON, P. A., Voluntary strength and fatigue. *J. Physiol. (Lond.)* 1954. 122. 553-564.
- NYSTRÖM, B., Mechanical and electrical responses to single shocks in developing cat leg muscles in tetanization. *Acta physiol. scand.* 1968 a. 74. 207-225.
- NYSTRÖM, B., Fibre diameter increase in nerves to "slow red" and "fast white" cat muscles during development. *Acta Neurol. Scandinavica*. 1968 b. 44. 265-294
- NYSTRÖM, B., Postnatal development of motor nerve terminals in "slow red" and "fast-white" cat muscles. *Acta Neurol. Scandinavica*. 1968 c. 44. 363-383
- NYSTRÖM, B., Histochemical studies of end plate bound esterase in "slow-red" and "fast-white" muscles during postnatal development. *Acta Neurol. Scandinavica*. 1968 d. 44. 295-312.
- NYSTRÖM, B., Post tetanic decararization in developing cat leg muscles. *Acta physiol. scand.* 1968. 74. 226-237
- NYSTRÖM, B., Effect of direct tetanization on twitch tension in developing cat leg muscles. *Acta physiol. scand.* 1968 f. 74. 319-339
- NYSTRÖM, B., Histochemistry of developing cat muscles. *Acta Neurol. Scandinavica*. 1968 g. 44. 403-414
- PROSKE, U. and P. M. E. WATZ, Properties of types of motor units in the medial gastrocnemius muscle of the cat. *Brain Res* 1974. 67. 89-101
- RANNEY R W and S. F. STREET, Absence of fatigue of the contractile mechanism in single motor units. *Fed. Proc.* 1942. 1. 70.
- RANVIER, L., De quelques faits relatifs à l'histologie et à la physiologie des muscles striés. *Annales Physiol. Norm. et Pathol.* 1874. 6. 1-15
- ROMANUL, F. C. A., Capillary supply and metabolism of muscle fibers. *Arch. Neurol.* 1963. 12. 65
- ROSENBLUTH A. and R. B. MORRISON, Curarization, fatigue and Wedensky inhibition. *Ann. N.Y. Acad. Sci.* 1937. 119. 236-256.
- SHAMARINA, N. M. Postnatal response of a single muscle fibre to indirect stimulation. *Soviet Physiol. USSR (Engl. Transl.)*. 1961. 47. 1145-1155
- STANDAERT F G Th. mechanisms of post tetanic potentiation in cat soleus and gastrocnemius muscles. *J. gen. Physiol.* 1964. 47. 987-1001
- WESTERMAN, R. A. B. M. LAWRIE, J. BAQUET, G. EDSTENHAGEN and D. PALLOT, Communication between nerves and muscles. Postnatal development in kitten hindlimb fast and slow twitch muscle. In *Handbook of Neurophysiology* Ed. H. H. Jasper, Plenum Publ. Corp., London. 1973. 255-291

*Preparation*

preparations were left with an intact arterial blood supply via the femoral artery. In these experiments a femoral artery was cannulated and connected to a pressure reservoir, containing an artificial perfusate (4-6 dextran-Tyrode solution or 4-10 Ficoll-Tyrode solution). The osmolarity of the perfusate was adjusted to the same level as the cat's own blood, i.e. usually around 300-310 mOsm/l. Long clamps alternately on the tubing from the reservoir and on the femoral artery, the perfusion could be rapidly shifted from blood to Newtonian fluid. By adjustments of a screw clamp on the artery or of the reservoir pressure, the inflow pressure could be exactly controlled.

In the preparations the femoral artery was divided and its peripheral end connected to two pressure arms, one containing 4 or 10 Ficoll-Tyrode solution, the other erythrocytes, suspended in the same Tyrode solution. The reason for using Ficoll instead of dextran solution as a vehicle for the erythrocytes was that their suspension stability was very good in Ficoll (the sedimentation rate was then less than 1% in 1 h) as contrast to the situation when erythrocytes are suspended in dextran, in which case the sedimentation rates are extremely high. Erythrocytes are obtained by centrifugation of heparinized arterial blood. During the bleeding large amounts of Ficoll were given to allow for effective coagulation. Local was collected in glass cylinders, containing glass beads, and shaken vigorously to get rid of the fibrin. The blood was centrifuged and the red cells resuspended in Ficoll, this was repeated 3 times and the last centrifugation the haematocrit was adjusted to 35-40%. The suspension was passed through a filter to remove any clots before it was placed in the pressure reservoir. The reservoirs containing the perfusates were placed in a water bath at 38°C. In these preparations, here the perfusion throughout occurred from pressure reservoirs, the femoral artery and vein were divided proximal to the site of cannulation, there was severance and the calf thus completely separated from the animal.

*Measurements of circulatory variables*

Infusion ( $P_i$ ) and venous outflow ( $P_v$ ) pressures were recorded with Statham pressure transducers, fully calibrated and repeatedly checked during the experiment. Recordings are made on Grass graph volume (weight) changes are recorded by means of modified force displacement transducers, sensitivity of the recording system was set so that 0.1 ml (0.1 g) made a deflection of around 10 mm on the polygraph — i.e. accuracy of blood and other perfusates was somewhat less than Oswald's method. The osmolarity of the perfusates as measured in an osmometer (Advanced Instruments Inc.)

*Mathematical procedures*

Perfusion was usually perfused with the artificial, cell-free, oxygenated perfusate. Great care was taken to maintain arterial inflow pressure low throughout to avoid oedema formation in the interstitial and vascular bed. Early in the experiment the inflow and outflow tubings were temporarily clamped so that no circulatory exchange could occur thus checking for leakage from the polygraph, bleeding and evaporation from the preparation and drift in the recording system. On resuming the perfusion,  $P_i$  and  $P_v$  were adjusted so that an isovolumetric (isogravimetric) situation was obtained. The capillary state in this situation,  $P_{C_0}$ , is then determined according to a modification of the technique by Pappas and Soto-Rubio (1948), earlier described in detail (Elam et al. 1974). First  $P_A$ , and then  $P_V$ , are separately elevated to levels causing identical  $P_i$  increments, to judge from the induced filtration rate, via the assumption of the pre-postcapillary resistance ratio, and hence  $P_{C_0}$  can be determined. The capillary volume coefficient (CFC) is subsequently determined by elevating  $P_i$  and  $P_v$ , and hence also  $P_{C_0}$  to the same amount, recording the filtration rate as volume or weight gain per time unit.

By adjusting  $P_i$  and  $P_v$  perfusate flow as then varied over fairly large ranges, although the highest values and flow were avoided except for short periods, to maintain oedema formation. The  $P_i$  value for each pair of  $P_i$  and  $P_v$  levels could be determined as the rate of volume (weight) change (from the isovolumetric state) and from the known CFC. To ensure that these deductions remained valid, the  $P_{C_0}$  and CFC were repeatedly measured during the perfusion period. — With knowledge of the volume flow  $P_i$  and  $P_v$  the pre- and postcapillary resistances ( $R_A$  and  $R_V$ , respectively) during perfusion with Newtonian fluid could be determined. The dependence of  $R_A$  and  $R_V$  on the mean arterial and venous perfusion pressures ( $P_i$ ,  $P_2$  and  $P_v$ ,  $P_{V2}$  respectively) could then be calculated.

After washing the above measurements with the cell-free perfusate, perfusion was switched to blood or erythrocyte suspensions.  $R_A$ ,  $R_V$ ,  $P_i$  and CFC were again determined in the isogravimetric state and during varying flow rates. In calculations of the respective  $R_A$  and  $R_V$  values from CFC and rate of weight

than the value obtained at maximal flows (Bäckström *et al.* 1971). These observations suggest that changes in viscosity are not a normally important determinant of blood and overall resistance, provided the rheological properties of the blood are not altered as in trauma, infection, shock, etc.

This does not mean, however, that changes in blood viscosity are always of importance in the control of circulatory homeostasis. For example, the anomalous properties of blood, meaning that its viscosity increases with increasing tube diameter decreasing flow rates suggest that effective blood viscosity would be higher in the wide post-capillary vessels than in the narrow precapillary vessels, and particularly so when reduced e.g. after neurogenic precapillary constriction. If this were true the higher viscosity would by its further relative increase, counteract the increase in preposition resistance ratio caused by the luminal changes and perhaps even increase capillary pressure as a net result. If so, these differences in effective blood viscosity within the pre- and post-capillary vascular compartments might even promote filtration and hence reduce the volume.

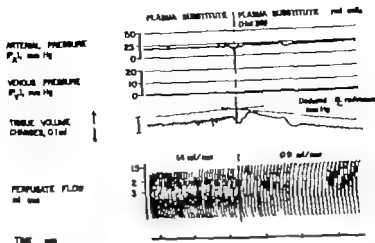
For these reasons it was considered of interest to estimate separately effective viscosity in the pre- and postcapillary vascular sections. This was done by comparing resistances in the two sections when they were perfused with either blood or a Newtonian non-corpuscular perfusate during conditions when vascular dimensions were rigidly controlled. This approach also allowed for a detailed analysis of the passive shifts in pre- and postcapillary resistances resulting from alterations of the transmural pressure. As the viscosity studies are concerned the results went, to our initial surprise, in the opposite direction to what was theoretically predicted as will be described below. — Part of this work has been reported earlier (Öberg 1974).

## Methods

Experiments were performed in 19 cats anaesthetized with chloralose (50–60 mg/kg b.w.). The calf muscle preparation, earlier described in detail (e.g. Djojosegito *et al.* 1970, Bäckström 1971) was used. Briefly the calf was isolated from the animal with exception of the femoral vein. A hole was drilled in the femur and the calf was plugged with cotton wool to a tight seal. Circulation from the calf to the bone marrow. Two subcutaneous branches to the femoral artery and calf muscles were cannulated, one to provide for measurements of arterial inflow pressure, the other for i.a. infusions of vasodilator drugs. A side branch of the femoral vein was used for measurements of venous outflow pressure. The femoral vein was cannulated proximally to the preparation and the venous outflow directed through a Doppler-recorder and recorded by an ordinate-integrator on a polygraph recorder. The blood (perfusate) flow was checked repeatedly by collecting the outflow in a graduated test tube. Venous outflow pressure could be varied by adjusting the height of the free outflow catheter above the preparation.

Sustained maximal vasodilatation in the preparation was induced by infusing intraarterial papaverine (1 mg/min) or nitroprusside sodium (50 g/min), dissolved in the same plasma substitute used for the perfusions (see below).

The preparation was then placed either in a plethysmograph for recording changes in tissue volume (e.g. Kjellmer 1964) or on a small balance for recording changes in tissue weight. When the calf muscle preparation was recorded the skin was cautiously removed with ligation of all its blood vessels and the muscles were protected from evaporation by a thin layer of silicone grease covered by a polyethylene envelope, and from cooling by a heating lamp.



2. The effect of changing perfusate from cell-free plasma substitute (6% Ficoll) to the same plasma substitute to which red blood cells were added to hematocrit of 39%. Note the reduction of flow despite unchanged pressure head when the cell suspension enters the vascular bed, reflecting an increased effective viscosity and the change of volume record from filtration to an absorption slope, indicating reduction in capillary pressure. This capillary pressure fall was estimated to amount to 3 mm Hg.

leading a gradual passive elastic recoil of the precapillary resistance vessels. Similarly precapillary resistance increases almost linearly until transmural pressure reaches 6–8 mm Hg but upon further pressure reductions disproportionate increases of venous resistance occur.

In all likelihood this latter phenomenon is due to increasing venous collapse, i.e. a change in venous cross-sectional geometry to an increasingly elliptical shape. In vivo experiments also illustrate that such a change of geometry starts when transmural pressure falls below 6–8 mm Hg (e.g. Öberg 1967). This marked increase of postcapillary resistance at low transmural pressures means that the pre- and postcapillary resistance ratio ( $R_A/R_V$ ) decreases sharply in this pressure range. This is demonstrated in the right part of Fig. 1.

The present data thus indicate that the postcapillary resistance is drastically increased by venous collapse when the mean distending pressure in this section falls below some 8 mm Hg. This phenomenon tends to prevent capillary pressure from falling to very low values following e.g. precapillary constriction, at least until such constrictions are so intense that a flow limitation of capillary absorption also ensues. In other words, the increasing post-collapse automatically tends to limit the extent to which a reflex capillary pressure fall can result in any effective fluid absorption into the vascular bed, which is clear when the  $R_A$  and  $R_V$  curves in Fig. 1 are considered.

#### Determinations of effective blood viscosity in pre- and postcapillary sections

In principle the same technique as earlier used (Dyosugito *et al.* 1970) was applied for the present determination of *in vivo* viscosity in the pre- and postcapillary vascular circuits; the flow resistances for "blood" and a Newtonian fluid in the two sections were compared, at identical vascular dimensions. The latter qualification was fulfilled by keeping

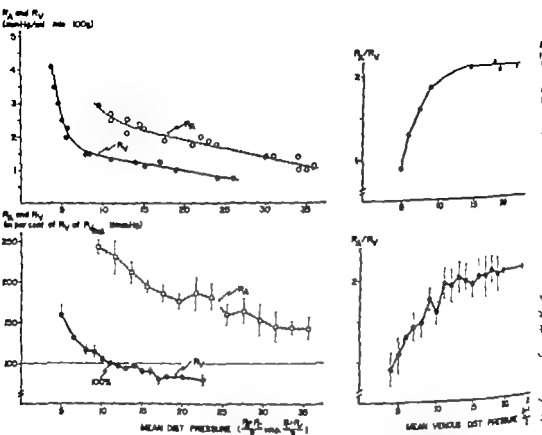


Fig. 1 The effect of varying transmural distending pressure on the precapillary ( $R_A$ ) and postcapillary ( $R_V$ ) resistances (left), and the influence of venous transmural pressure on the pre/post-capillary resistances ratio (right). The upper records are from one experiment, the lower diagrams show compiled data ( $\pm 3SE$ ) from 9 cats.

(volume) change. By comparing  $R_A$  and  $R_V$  during blood (o erythrocyte suspension) and perfusing the Newtonian perfusate (identical distending pressures (and hence vessel geometry) the effective blood viscosity in the pre- and postcapillary vascular compartments could be determined.

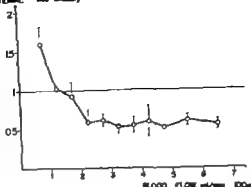
## Results

**1 Analysis of pressure-passive changes of flow resistance in pre- and postcapillary vessels**

While the transmural distending pressures were varied over a wide range, the flow resistances in the pre- and postcapillary vascular sections were determined from the pressure-flow and flow relation during perfusion with Newtonian fluids, *i.e.* for precapillary resistance ( $P_A - P_C/\text{flow}$ ), and for postcapillary resistance ( $P_O - P_V/\text{flow}$ ). The capillary pressure ( $P_C$ ) was, determined, as described above, from the prevailing rate of filtration or absorption and the CFC value, which data allowed for calculations of the deviation of the actual  $P_C$  from the isogravimetric  $P_O$  level ( $P_{C_0}$ ). The relation between transmural pressure and resistance in the pre- and postcapillary sections is shown in Fig. 1 both as average value from 9 compiled expts. and data from one representative experiment. As seen, precapillary resistance increases in an essentially linear fashion upon reduction of the transmural pressure



VENOUS  $\frac{\text{viscosity}}{\text{ARTERIAL}}$



4. The question between postcapillary precapillary viscosity at venous flow. The data are plotted according to each symbol represent the mean  $\pm$  SD, that the venous viscosity exceeds the arterial viscosity only in the very lowest flow.

The effective blood viscosity for the entire vascular bed decreases at higher flows (e.g. Jonsgaard *et al.* 1970). Calculated effective precapillary viscosity on the other hand, does change or even increases with increasing flow i.e. if anything it alters in the opposite direction to venous and "total" blood viscosities. One consequence of these opposite changes of venous and arterial blood viscosities with changes in flow is that the ratio between venous and arterial viscosities remains below 1 over almost the whole range of flows (Fig. 4). It is only when the flow rate is around or below 1 ml/min  $\cdot$  100 g that the theoretical prediction of a higher viscosity in the wide bore postcapillary vessels is true.

Results from one illustrative experiment are demonstrated in Fig. 5 in which  $R_A$  and  $R_V$  relations were analysed also at high pressures and flows. With Newtonian fluid ("plasma") perfusion  $R_A$  and  $R_V$  decreases with increasing pressures and flows. The quotient  $R_A/R_V$  also increases when the pressures are raised from low levels, however in the higher pressure range this ratio remains largely constant throughout.

It is also seen that precapillary blood viscosity does not decrease with flow rate, at least not in the same proportion as "total" viscosity and venous viscosity: thus the ratio between arterial and venous viscosities increases at high levels of blood flow (20 ml/min  $\cdot$  100 g and above) (not shown in Fig. 4, since only fairly low flow levels were investigated in these experiments). Consequently  $R_A/R_V$  is considerably higher during "blood perfusion" than during plasma perfusion. As a result  $P$  is significantly lower blood perfusion, and particularly at higher flows where, according to Fig. 5, the presence of red cells leads to a reduction of 7 mm Hg for the same  $P$  and  $P$  levels and at a blood flow around 20 ml  $\cdot$  100 g.

Comparisons of the haemodynamic (osmometric) capillary pressures for Ficoll and Ficoll with suspended erythrocytes

It is well known that together with the oxygen delivery in the capillaries a certain hyperosmolality arises within the red cells which as a result is due to a net influx of water. This means that the presence of red cells in the perfusate, which give up part of their oxygen to the tissues, adds certain reabsorption forces to the ordinary colloid osmotic forces. The

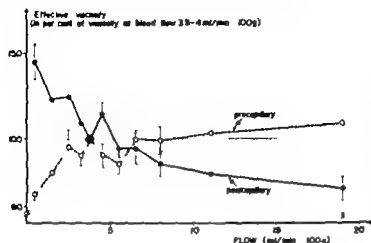
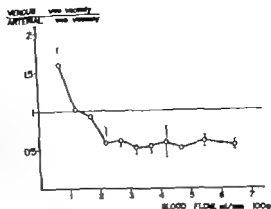


Fig. 3. Alterations of pre- and postcapillary resistance at blood flow. Data from experiments, the data has been normalized according to the flow rate, thus represent mean values. Number of observations per group are given above axis. To normalize the viscosity value at a flow of 3.5 and 4 ml/min is set at 100%.

mean distending pressure in the two sections as identical as possible for the experiment while active dimensional shifts due to smooth muscle activity were avoided by administering huge doses of vasodilator drugs. As mentioned, due to the anomalous viscosity of blood one would *a priori* expect a higher effective blood viscosity in the widebore postcapillary vessels and therefore a fall in pre/postcapillary resistance ratio, when shifting from Newtonian fluid to "blood" perfusion. This would show up as a  $P_0$  increase when blood started, provided arterial inflow and venous outflow pressures were kept constant. If the colloid osmotic properties of the Newtonian fluid and the "blood" were identical, an increased  $P_0$  should reveal itself as a net filtration, i.e. as a net gain of volume, of the preparation.

However as it turned out quite the opposite phenomenon regularly occurred. When shifting from Newtonian fluid perfusion to "blood" perfusion there was a net filtration, indicating a  $P_0$  reduction and, consequently a relatively more pronounced fall of precapillary than of postcapillary resistance. This is illustrated in Fig. 2, when perfusion of the calf muscle preparation is suddenly changed from 6% Ficoll to "blood", i.e. the same 6% Ficoll solution with suspended erythrocytes to a hemoglobin solution. Despite constant  $P_A$  and  $P_V$  levels and the same colloid osmotic pressure, the "blood" leads to a clearcut net absorption of tissue fluid, i.e. to a  $P_0$  reduction. The absorption rate and the CFC value, determined for "blood" later in the experiment, the actual  $P_0$  fall was calculated as approximately 3 mm Hg at this low flow level.

The phenomenon shown in Fig. 2, which was always observed when shifting from Newtonian perfusion to "blood" perfusion, implies that effective blood viscosity is higher in precapillary than in the postcapillary vessels, which is contrary to what is theoretically predicted. The effective blood viscosity in pre- and postcapillary vessels was similarly determined in 8 experiments at varying "blood" flows and the data are shown in Fig. 3. To normalize the data, which differed considerably between experiments due to different hematocrits and viscosities of the suspension medium, the values for different flow levels are expressed as per cent of the viscosity value at a flow between 3.5 and 4 ml/min 100 g. It is seen that effective venous viscosity decreases with increasing "blood



4. The quotient between postcapillary precapillary viscosity at various flow rates. The data are classed according to each symbol represent the mean  $\pm$  SD that the venous viscosity exceeds the arterial viscosity only in the very lowest flow rates.

the effective blood viscosity for the entire vascular bed decreases at higher flows (e.g. Ojimoto *et al.* 1970). Calculated effective precapillary viscosity on the other hand, does not change or even increases with increasing flow i.e. if anything it alters in the opposite direction to "venous" and "total" blood viscosities. One consequence of these opposite changes of venous and arterial blood viscosities with changes in flow is that the ratio between venous and arterial viscosities remains below 1 over almost the whole range of flows (Fig. 4). It is only when the flow rate is around or below 1 ml/min 100 g that the theoretical prediction of a higher viscosity in the wide bore postcapillary vessels is true.

Results from one illustrative experiment are demonstrated in Fig. 5 in which  $R_p$  and  $R_v$  relations were analysed also at high pressures and flows. With Newtonian fluid ("plasma") perfusion  $R_A$  and  $R_p$  decreases with increasing pressures and flows. The quotient  $R_A/R_v$  also increases when the pressures are raised from low levels, however in the higher pressure range this ratio remains largely constant throughout.

It is also seen that precapillary "blood" viscosity does not decrease with flow rate, at least not in the same proportion as "total" viscosity and venous viscosity: thus the ratio between arterial and venous viscosities increases at high levels of blood flow 20 ml/min 100 g and above (not shown in Fig. 4 since only fairly low flow levels were investigated in these experiments). Consequently  $R_A/R_p$  is considerably higher during blood perfusion than during plasma perfusion. As result P is significantly lower blood perfusion, and particularly at higher flow where, according to Fig. 5, the presence of red cells leads to a reduction of 7 mm Hg for the same  $P_p$  and  $P_v$  levels and at a blood flow around 20 ml/min 100 g.

Comparisons of the isovolumetric (isogravimetric) capillary pressure for Ficoll and Ficoll with suspended erythrocytes

It is well known that together with the oxygen delivery in the capillaries a certain hyperosmotic stress arises within the red cells which as a result swell due to a net influx of water. This means that the presence of red cells in the perfusate, which give up part of their oxygen to the tissues, adds a certain reabsorption force to the ordinary colloid osmotic forces. The

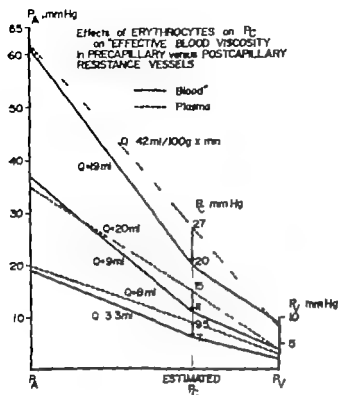


Fig. 5

	$P_A$	$P_V$	$P_C$	Flow	$R_A$	$R_V$	$R_A/R_V$	$Visc_{tot}$	$Visc_A$	$Visc_V$	$Visc_A/Visc_V$
"Plasma" (Ficoll 10%)	20	3	9.5	8	1.3	0.8	1.63	1.0	1.0	1.0	1
	35	4	15	20	1.0	0.55	1.82	1.0	1.0	1.0	1
	62	8	27	42	0.83	0.45	1.84	1.0	1.0	1.0	1
"Blood" (Ficoll 10%)	19	2	7	3.3	3.65	1.5	2.45	2.45	2.8	1.9	1.5
	37	4	11	9.0	2.9	0.78	3.70	3.35	2.9	1.42	2.05
+ erythrocytes to Hct 40%	61	9	20	19.0	2.15	0.58	3.70	2.10	2.6	1.28	2.05

Data from one experiment presented both as simplified pressure drop profiles along the vascular bed and as a tabular form. At different pressures ( $P_A$ ,  $P_V$ ,  $P_C$  in mm Hg) and flows (in ml/min/100 g), Newtonian fluid and cell suspension perfusions are compared with respect to their effects on pre- and postcapillary resistances ( $R_A$ ,  $R_V$ ), on "total" viscosity ( $Visc_{tot}$ ) and on pre- and postcapillary viscosities ( $Visc_A$ ,  $Visc_V$ ). In all, viscosity of the Newtonian plasma substitute is set at 1.0.

Importance of this reabsorption force, exerted by the red cells, has been discussed from quantitative point of view by Tybjaerg Hansen (1961). This mechanism is, according to Tybjaerg Hansen's calculations, capable of transporting large amounts of fluid from the tissues to the lungs. However during normal "resting" situation in skeletal muscle, the reabsorption force can be calculated to correspond to less than 1 mm Hg transcapillary pressure difference, but evidently this figure must increase considerably during increased blood flow and tissue oxygen uptake. At any rate, such an osmotic "Tybjaerg Hansen effect" of red cells must be considered in the present studies. The change from filtration to absorption when shifting from Ficoll perfusion to Ficoll + erythrocyte perfusion (cf Fig. 2) can thus, to an unknown extent, be due to a "Tybjaerg Hansen effect" and not only to a decrease of hydrostatic capillary pressure. The capillary pressure may thus in fact be higher than pre-

d from the rate of absorption and the calculated value for pre/postcapillary resistance consequently erroneously high.

order to confirm experimentally in an *in vivo* preparation the existence of such an active resorption force, exerted by the red cells, and to get a rough idea of its magnitude for the present experimental set-up, the  $P_{O_2}$ -values for Ficoll and Ficoll + erythrocytes carefully and repeatedly determined in 4 animals. It was then found that the average was  $0.63 \pm 0.07$  mm Hg higher when erythrocytes were added to the Ficoll passing the star bed of the resting calf muscles. In other words, to establish a complete balance between filtration and absorption forces,  $P_{O_2}$  must be 0.63 mm Hg higher in the presence of cells, implying that the red cells exerted an absorption force of this magnitude. Thus, the calculated absorption force of 3 mm Hg, required to produce the rate of fluid absorption in Fig. 2, only 0.63 mm Hg, or around one fifth, can be ascribed to a "Tybjaerg-Hansen phenomenon". In the present study with low flows and low  $O_2$ -consumption in skeletal muscle the "Tybjaerg-Hansen phenomenon" therefore does not seem to play an important role, and this factor was consistently disregarded in the calculations of  $R_{A_2}$ , etc., during "blood" perfusion. As will be discussed below the situation must be different during intense muscle exercise.

### Discussion

postcapillary flow resistance is one important factor determining the capillary pressure and hence transcapillary fluid movements and plasma volume. Few attempts have, however, been made to analyze how this postcapillary resistance is controlled. It has quite generally been taken for granted that active shifts in venous vessel tone constitute the most important factor influencing the flow resistance in this section (e.g. Hadjiminas and Öberg 1964). However, some scattered observations from earlier studies suggest that also other mechanisms are capable of affecting the resistance in the venous section. Thus, the remarkable drop of the capillary pressure when arterial pressure falls (cf. Folkow and Öberg 1964; Järhult and Mellander 1974), can hardly be explained by an autoregulatory reduction of the precapillary resistance alone but seems to require also an increase in postcapillary resistance. In the present series of experiments two passive mechanisms have been analysed which might reasonably be expected to affect the postcapillary resistance, namely pressure-passive alterations of vessel geometry and postcapillary blood viscosity.

The results of these studies have shown that, as expected, the flow resistance increases in both pre- and postcapillary sections when transmural distending pressure falls, provided that active adjustments of smooth muscle tone are eliminated by maintaining the vascular bed maximally dilated throughout. This increase of flow resistance in both the pre- and postcapillary sections, following e.g. stepwise reductions of arterial inflow pressure, occurs roughly in parallel so that no drastic shifts in the ratio between the pre- and postcapillary resistances occur as long as the average venous transmural pressure does not fall below 8-9 mm Hg. Below this pressure level venous resistance increases out of proportion to a further decrease in distending pressure. This very marked increase of postcapillary resist-

ance, which in all probability depends on an increasing degree of venous collapse (Öberg 1967), leads to a decrease of the pre/postcapillary resistance ratio ( $R_A/R_V$ ). This ratio, which at higher pressure levels amounts to approximately 2/1 in the maximally distended muscle bed when perfused with an oxygenated Newtonian fluid, falls towards 1/1 as the venous transmural pressure falls below 6–8 mm Hg. Thus  $R_A/R_V$  reduction constitutes one inherent passive mechanism which prevents the capillary pressure from falling to low levels along with e.g. a reduction of the arterial pressure.

These pressure-passive changes of  $R_A$  and  $R_V$  illustrate the uncertainties inherent in determinations of  $R_A/R_V$  and  $P_{CO}$  at least when the original method of Pappenheimer & Soto-Rivera (1948) is employed, implying a simultaneous increase of  $P_V$  and decrease of  $P_A$  with maintenance of an isogravimetric state. It is evident that these manoeuvres may cause an increase of  $R_A/R_V$  i.e. a change of the variable one wishes to measure. This disturbance becomes particularly marked if the procedures are performed at low venous transmural pressures. The inherent error in the determination of  $R_A/R_V$  and  $P_{CO}$  is considerably reduced in the present modification of the technique of Pappenheimer & Soto-Rivera, where both  $P_A$  and  $P_V$  and hence  $R_A$  and  $R_V$  are changed in the same direction and only to a small extent, thus tending to minimize the unavoidable, experimenter-induced shifts in  $R_A/R_V$ .

The background to the unexpected finding of a higher "effective" blood viscosity in precapillary than in the postcapillary vessels is not fully clear. Theoretically the former would be expected since effective viscosity of anomalous fluids, like blood, is lower the smaller the tube radius and the greater the linear flow rate. A possible explanation may follow. The proximal, quite narrow sections of the true capillaries must be considered part of the precapillary resistance section (as defined by Eliassen *et al.* 1974) because of the result of the increasing porosity of the capillary membrane towards the wider distal sections and in the venules (e.g. Zweifach and Intaglietta 1969), they are placed proximal to the equilibrium point for the filtration-absorption process along the exchange vessels. Furthermore, it is known that a certain deformation of the red cells occurs when they are squeezed through the metarterioles and capillaries which in proximal parts have diameters of 4–5  $\mu\text{m}$  in skeletal muscle (e.g. Eriksson and Myrhaug, 1972). Thus a certain force is required to cause this "bending" of the cells (cf. Braasch, 1971), and this will take place particularly in the proximal end of the capillaries, while the wider downstream exchange vessels hardly call for any erythrocyte bending or friction against the wall. Even if the force required to deform erythrocytes is relatively small, it will nevertheless imply an energy loss and therefore an added pressure fall in these narrow metarteriolar-proximal capillary sections. The higher the blood flow and hence the more red cells pass these proximal capillary sections per unit time, the more pronounced the regional pressure fall as a result of this "bending flow".

This latter feature showed up in the present study as an almost constant, or even slightly elevated "apparent" viscosity in precapillary vessels when flow increased, while in the distal situation one would have expected rather a reduction of viscosity due to the anomalous viscous properties of blood with a reduced viscosity at high shear rates. Thus, what is measured as a "precapillary blood viscosity" is not only a genuine viscosity but also a pressure

the energy losses inherent in the deformation and wall friction of red cells when they pass through the most narrow sections of the vascular bed, *i.e.* the distal metarterioles and terminal parts of the capillaries. The tendency for "true" blood viscosity to decrease with increasing flow rate is thus offset in the most distal segment of the precapillary vessels by the increased energy losses. The net effect is an almost unchanged, or sometimes even increased effective precapillary viscosity along with increases of flow.

The functional implication of this considerable increase of precapillary flow resistance when red cells are added to the perfusate was demonstrated in Fig. 5, and may be further illustrated by the following example. Assume in a resting muscle a  $P_A$  of 100 mm Hg, a  $P_V$  of 5 mm Hg and a  $P$  value around 15–20 mm Hg (Elaassen *et al.* 1974), *i.e.* the  $R_A/R_V$ -ratio is 5–8/1. With heavy exercise and maximal vasodilatation, the  $R_A/R_V$ -ratio would decrease, according to the present study to less than 2/1 if no erythrocytes are present. With the same  $P_A$  and  $P_V$  levels,  $P$  would then rise to 35–40 mm Hg, *i.e.* by 20–25 mm Hg. This added  $P$  rise, in combination with the high CFC values in exercising muscles (0.04–0.05), could mean extensive filtration losses. The presence of red cells implies, however, that the  $R_A/R_V$ -ratio would decrease to only 3.5–4/1 at maximal flow values (Fig. 5). In this situation, with  $P_A$  at 100 mm Hg and  $P_V$  at 5 mm Hg,  $P$  would assume a value around 25–27 mm Hg.  $P$  would increase by at most some 10 mm Hg. Thus, by keeping  $R$  relatively low at maximal vasodilatation and high flows, the red cells are capable of keeping  $P$  low thereby reducing the filtration losses considerably. Moreover the red cells also help to keep fluid within the vascular system in heavily exercising muscles by the earlier mentioned splanchnic Hansen effect. Even if the reabsorption force exerted by this mechanism, must be rather small in the resting state, it must increase in potency 20–30-fold in heavily exercising skeletal muscles since it is known that their oxygen extraction then often increases 5-fold and their blood flow 10-fold. The red cells thus contribute in two important ways to the preservation of the intravascular fluid volume in *e.g.* heavy exercise, by helping to counteract the fluid transfer caused by the often marked regional increases in tissue osmolarity (Mellander *et al.* 1967; Lundvall 1972).

The idea which initiated the present study was that postcapillary blood viscosity was likely to be higher than precapillary blood viscosity since *in vitro* studies have made it clear that blood viscosity increases with increases of tube dimensions and with decreases in linear flow rate. If applicable to the normal vascular bed, such a regional viscosity difference would tend to decrease  $R_A/R_V$  and hence increase  $P$ , perhaps even to the extent that filtration losses might ensue at low flow rates. The experimental findings were, however, contrary to these theoretical predictions. Postcapillary blood viscosity was then decidedly lower than precapillary blood viscosity over nearly the entire flow range. Only at the very lowest flows, below some 1 ml/min (100 g) (as may occur in severe shock), did postcapillary blood viscosity approach and eventually exceed precapillary blood viscosity. In all other situations the most important consequence of the higher precapillary blood viscosity *in situ* seems to be a lower capillary pressure and consequently help to prevent filtration losses from the cardiovascular system.

The present study has been supported by grants from the Swedish Medical Research Council (No. B75-16-11A, B75-14V-44-11). All fluids generously covered part of the expenses for technique.

## References

- BAECKSTRÖM, P. B. FOLKOW, E. HENDRICK, B. LÖFVING and B. ÖBERG, Effects of vasoconstrictor blood viscosity *in vivo*. *Acta physiol. scand.* 1971. 81. 376-384.
- BRAASCH, D. Red cell deformability and capillary blood flow. *Physiol. Rev.* 1971. 51. 679-701.
- DJONOGUPTA, A. M. B. FOLKOW, B. ÖBERG and S. WHITE, A comparison of blood viscosity *in vivo* and in a vascular bed. *Acta physiol. scand.* 1970. 78. 70-84.
- ERIKSSON, E. and R. MYRHAGE, Microvascular dimensions and blood flow in skeletal muscle. *Acta physiol. scand.* 1972. 86. 11-22.
- ELIASSEN, E. B. FOLKOW, S. M. HILTON, B. ÖBERG and B. RIPPE, Pressure-volume characteristics of the interstitial fluid space in the skeletal muscle of the cat. *Acta physiol. scand.* 1974. 90. 583-593.
- FOLKOW, B. and B. ÖBERG, Autoregulation and basal tone in consecutive vascular sections of the skeletal muscle in reserpine-treated cats. *Acta physiol. scand.* 1961. 53. 105-113.
- HADJIDEMAS, J. and B. ÖBERG, Effects of carotid baroreceptor reflexes on basal tone in skeletal muscle and intestine of the cat. *Acta physiol. scand.* 1968. 72. 518-532.
- JÄRNVALL, J. and S. MELLANDER, Autoregulation of capillary hydrostatic pressure in skeletal muscle: regional arterial hypo- and hypertension. *Acta physiol. scand.* 1974. 91. 32-41.
- JÄRNVALL, J. The effect of exercise on the vascular bed of skeletal muscle. *Acta physiol. scand.* 1973. 89. 18-30.
- LUNDVALL, J. Tissue hyperosmolality as a mediator of vasodilatation and transcapillary fluid transfer in exercising skeletal muscle. *Acta physiol. scand.* 1972. Suppl. 379.
- MELLANDER, S., B. JOHANSSON, S. GRAY, O. JOHANSSON, J. LUNDVALL and B. LUNDA, The effects of osmolality on intact and isolated vascular smooth muscle. Possible role in exercise hypertension. *Circulation* 1967. 4. 310-322.
- ÖBERG, B. Effects of cardiovascular reflexes on net capillary fluid transfer. *Acta physiol. scand.* 1971. Suppl. 49.
- ÖBERG, B. The relationship between active constriction and passive recoil of the arterial smooth muscle. *Acta physiol. scand.* 1967. 71. 233-247.
- ÖBERG, B. Aspects on the control of the capillary filtration process. *Cardiologia* 1974. Suppl. 28.
- PAPPENHEIMER, J. R. and A. SOTO-RIVERA, Effective osmotic pressure of the plasma proteins and quantities associated with the capillary circulation in the hindlimbs of cats and dogs. *Amer. J. Physiol.* 1948. 152. 471-491.
- TYLER, H. G. and A. HANSEN, Osmotic pressure effects on the red blood cells. Possible physiological significance. *Nature (Lond.)* 1961. 190. 504-508.
- WHITTAKER, S. R. F. and P. R. WINTON, The apparent viscosity of blood flowing in the isolated hindlimb of the dog and its variation with corpuscular concentration. *J. Physiol. (Lond.)* 1933. 72. 339-354.
- ZWEIFACH, B. W. and M. INTAGLIETTA, Mechanics of fluid movement across single capillaries in the microcirculation. *Rev.* 1969. 1. 83-101.



## Kinetics of the Glomerular Ultrafiltration in the Rat Kidney A Theoretical Study

By

Ö. KALLÉNG, L. O. LINDSOM, H. R. ULFENDAHL and M. WOLGAST

Received 18 April 1975

### Abstract

KALLÉNG, Ö., L. O. LINDSOM, H. R. ULFENDAHL and M. WOLGAST: *Kinetics of the glomerular ultrafiltration in the rat kidney. A theoretical study.* Acta physiol. scand. 1975. 95: 191-200.

glomerular filtration process as evaluated theoretically from micropuncture data obtained from tape-Dewey rats. The hydrostatic pressure in the glomerular capillaries and Bowman's space, osmotic pressure in systemic plasma gave the net driving force at the proximal end of the glomerular artery. From the single nephron filtration fraction the mean net driving force over the glomerular capillary was calculated to be 20 mm Hg during normotension, decreasing to 12 mm Hg during perfusion pressure of 80 mm Hg. The hydraulic permeability for one glomerulus was 0.7-0.8 ml/min 100 g b.wt. mm Hg. Pressures at the distal end of the glomerular capillaries were 13 and 6 mm Hg under the above two pressures, indicating non-equilibrium of the filtration process at the end of the glomerular capillary. It shows that the glomerular filtration rate is mainly influenced by the driving pressure. During hypotension an increased plasma flow dependency was evident. Brenner *et al.* local filtration equilibrium and plasma flow dependent glomerular filtration rate in anesthetized Wistar rat strains. The discrepancy between our results and theirs due to the low glomerular plasma flow and hydrostatic pressure in the Wistar rats. Concluded from our results that both pre- and postglomerular resistances may influence the glomerular filtration rate and glomerular plasma flow independently.

The kinetics of glomerular ultrafiltration have been the object of keen interest for some years, and the variables involved in the process will determine not only the fluid balance with respect to filtration but also, indirectly, the absolute tubular fluid reabsorption, at least where the proximal tubules are concerned. The circulatory factors operative in the renal vascular system thus play a cardinal part in the control of the extracellular and plasma volumes and thereby also in the control of cardiac output and systemic blood pressure.

Essentially two modes of experimental approach have been used in evaluating the glomerular filtration process. In the earliest approach the kinetics of glomerular ultrafiltration were examined by studying the transport of molecules of different sizes (Bott *et al.* 1941, Vallboom 1954, Chirard 1952, Pappenheimer *et al.* 1951). The membrane characteristics were determined, by means of the equations presented by Landis and Pappenheimer (1943).

In more recent studies by Lambert and coworkers (Lambert *et al.* 1972, Verniory *et al.* 1974) the theoretical basis of the sieving equations was re-examined (Solb 1968 Verniory *et al.* 1973) and applied to the glomerular ultrafiltration process in the kidney.

A direct approach involving determinations of driving forces and fluid flow over membrane in a single glomerulus was first presented by Brenner and co-workers (1971, 1972). In these studies direct punctures of the glomerular capillaries and Bowman's space were carried out on a unique mutant Wistar strain with superficially located glomeruli. In these animals the filtration fraction could also be determined from small samples of blood obtained from large welling points on the kidney surface. The results indicated a comparatively low glomerular capillary pressure and a low single glomerular plasma flow. A mathematical computer based evaluation revealed that an approximate filtration equilibrium is attained somewhere within the glomerular capillary tree and that the filtration is highly dependent on plasma flow.

The present evaluation of the glomerular ultrafiltration process is based on data obtained from normal full-grown male Sprague Dawley rats investigated by Källskog *et al.* (1975). In the latter study the occurrence, though rare, of superficially located glomeruli was sufficient for an estimation of the hydrostatic pressure operative over the glomerular membrane. In addition continuous sampling of blood from the welling points allowed calculation of colloid osmotic pressure in the distal end of the glomerular capillaries.

#### Experimental data

These were taken from Källskog *et al.* (1975). The glomerular capillary pressure as obtained by direct punctures was  $60.6 \pm 3.2$  mm Hg (mean  $\pm$  S.D.). The pressure in Bowman's space was  $16.0 \pm 0.8$  mm Hg which was 2.5 mm Hg higher than that in the proximal tubule. The hydrostatic pressure difference over the glomerular membrane was thus 46.6 mm Hg. With a plasma protein concentration (Källskog & Wolgast 1973) the colloid osmotic pressure of systemic plasma is 20.3 mm Hg. The net driving force at the proximal end of the glomerulus was calculated to be 6.3 mm Hg.

The pressure difference calculated from the top-flow pressure and the proximal tubular pressure according to Geriz *et al.* (1966) was 24.2 mm Hg in one of the series studied and 46.2 mm Hg in the other, thus in complete accordance with that obtained from direct punctures. During hypotension (mean pressure of 80 mm Hg) the pressure difference decreased to 18.3 mm Hg.

The filtration fraction as estimated from small blood samples withdrawn from the distal ends of efferent arterioles (welling points) was  $0.27 \pm 0.06$  (mean  $\pm$  S.D.) during normotension, changing non-significantly to  $0.48 \pm 0.03$  (mean  $\pm$  S.D.) at 80 mm Hg.

The single nephron glomerular filtration rate (SNGFR) was  $14.1 \pm 1.9$  nl/min/100 g b.wt. (the rats weighed about 300 g) under control antidiuretic conditions, decreasing to  $10.4 \pm 2.4$  nl/min/100 g b.wt. at 80 mm Hg. The single nephron plasma flow was calculated to be  $52.2 \pm 7.0$  nl/min/100 g and  $37.1 \pm 5.6$  nl/min/100 g under normotensive and hypotensive conditions, respectively.

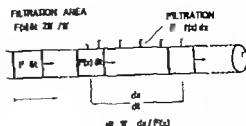
The blood flows would be about twice these figures, as the systemic hematocrit was 0.50. The mean arterial pressure averaged about 120 mm Hg. The pressure drops over the pre- and postglomerular vessels would be essentially the same. The arteriolar resistance was calculated to be approximately 0.6 mm Hg/100 g, and the vascular conductance thus  $1.7$  l/min/mm Hg/100 g b.wt.

#### Evaluation of the glomerular ultrafiltration process

The glomerular capillaries are assumed to constitute a cylindrical tube with a constant length  $x_0$  and a radius  $r$  (Fig. 1). The permeability is uniform along the capillary.

During a certain time interval,  $\Delta t$ , a volume segment passes into the capillary system. A

1. Hypothetical model of the glomerular capillaries assumed to constitute cylindrical tubes. The fluid filtered from the volume segment has passing from to + governed by the surface area of the segment of the glomerular membrane and the time of passage over the distance  $dx$ .



Along the capillary the volume of the segment is  $F dt$ , and at position  $x$  within the capillary volume is  $F(x) dt$ ,  $P$  is the plasma flow at the beginning of the capillary and  $F(x)$  the flow at position  $x$ . The surface area of the segment facing the capillary wall is then,

$$\text{Surface area} = F(x) dt \cdot 2\pi r / W$$

Eq 1

When passing over a distance  $dx$  a certain amount of fluid will be filtered as determined by the net driving force, the permeability characteristics of the glomerular membrane and time of passage,  $dt$ .

The net driving force is in turn determined by a) the hydrostatic pressure within the glomerular capillary (this is assumed to be constant at all positions in the capillary bed), b) the hydrostatic pressure within Bowman's space, and c) the colloid osmotic pressure of glomerular capillary blood. The latter factor can be calculated from the protein concentration with the aid of the formula given by Landis and Pappenheimer (1963)

$$2.1 C^2 = 0.16 C^3 + 0.09 C^2$$

Eq 2

where  $C$  is protein concentration at any position within the capillary is then calculated from the concentration in arterial plasma,  $C_0$ , and the filtration fraction. The increase in filtration volume per length unit is denoted  $f(x)$ , and the increase over the distance  $dx$  is thus  $f(x)dx$ . The total filtration fraction produced up to position  $x$  is accordingly  $\int_0^x f(x)dx$ . The protein concentration can then be expressed as  $C_0(1 - \int_0^x f(x)dx)$ . The oncotic pressure is:

$$2.1 \left[ \frac{C_0}{1 - \int_0^x f(x)dx} \right] = 0.16 \left[ \frac{C_0}{1 - \int_0^x f(x)dx} \right] + 0.09 \left[ \frac{C_0}{1 - \int_0^x f(x)dx} \right]$$

Eq. 3

Fig. 2 the pressure is related to the filtration fraction for 5%, 6% and 7% protein concentrations, where 6% is the concentration found during antidiuresis in the experimental rats. In the following, Eq. 3 is denoted

$$R \left( \int_0^x f(x)dx \right)$$

The net driving force on volume segment located at  $x$  is obtained as.

$$P - R \left( \int_0^x f(x)dx \right) - P_0$$

Eq 4

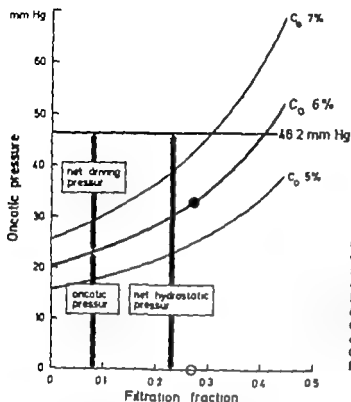


Fig. 2. Relation between filtration and plasma oncotic pressure at the end of the glomerular capillaries refers to various protein contents of systemic plasma. The oncotic pressure is calculated from the formula  $C = (1 - \text{filtration fraction}) \times 2.1 \text{ C. S.W.C.} + 0.009 \text{ C}^2$ . The circle gives the end-oncotic pressure at a filtration fraction 0.27 and indicates a residual net driving force of about 13 mm Hg.

where  $P_{\text{glom}}$  is the glomerular capillary pressure and  $P_{\text{down}}$  the pressure in Bowman's space. The arithmetic sum of these two pressures was 46.3 mm Hg and can be seen in Fig. 2.

The passage time for the volume segment over the distance  $dx$  is calculated (see Fig. 1) dividing the volume of the capillary system between  $x$  and  $x + dx$  by the actual flow  $F$  at position  $x$ , as follows:

$$\text{Passage time} = \pi r^2 dx / F(x) \quad (4)$$

The volume of fluid filtered in the segment  $x$  to  $x + dx$  is then obtained by multiplying the volume of the segment when it enters the glomerulus,  $F dt$ , by the filtration fraction increase  $f(x)dx$  produced during the passage from  $x$  to  $x + dx$ . This volume will be denoted as

$$F dt f(x) dx = \left[ P_{\text{glom}} - P_{\text{down}} - \pi \left( \int_0^x f(x) dx \right) \right] K \frac{2\pi r F(x) dt}{\pi r^2} \frac{\pi r^2 dx}{F(x)}$$

filtered volume = net driving force  $\times$  permeability  $\times$  surface area  $\times$  passage factor time

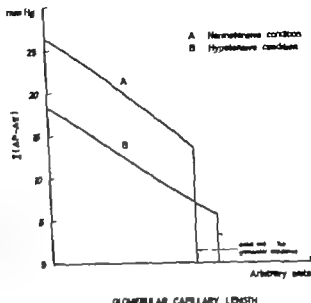
$$\text{or} \quad F f(x) dx = \left[ P_{\text{glom}} - P_{\text{down}} - \pi \left( \int_0^x f(x) dx \right) \right] K 2\pi r dx \quad (5)$$

where  $K$  is a permeability factor

The total filtered volume up to distance  $x$  is then obtained by integrating the equation from  $x = 0$  to  $x = x$

$$F \int_0^x f(x) dx = K 2\pi r \int_0^x \left[ P_{\text{glom}} - P_{\text{down}} - \pi \left( \int_0^x f(x) dx \right) \right] dx \quad (6)$$

3 Relation between net driving pressure and glomerular capillary length under normo- (A) and hypo- (B)-tensive conditions. Filtration equilibrium is reached under these conditions.



equation is easily solved graphically. For this purpose a series of filtration fractions 0.01, 0.03-0.27 are assumed. The driving forces valid when the filtration fractions are 0.01, 0.02 and so on are then calculated from Eq. 2 and 3 and are shown in Fig. 2 (curve 6%). Integration boundaries  $0-x_1, x_1-x_2, x_2-x_3$ , etc. are then calculated. This is performed by multiplying the mean net driving force during a particular interval by a length that will give constant filtration volume. The first length, where a filtration fraction of 0.01 is produced, is placed as one length unit. During the next interval (filtration fraction increases from 0.01 to 0.02) the mean net driving force is somewhat smaller and the length  $x_1-x_2$  is proportionately longer. The procedure is repeated until a total filtration fraction of 0.27, i.e. that found experimentally, is reached. In Fig. 3 the relation between the glomerular capillary length (arbitrary units) and the net driving force is depicted. The upper curve represents normal anisotensive conditions and the lower curve hypotensive conditions of about 80 mm Hg. The hydraulic permeability factor  $K$ , was considered to be unchanged. The ratios between the flows,  $F$ , under the two conditions were obtained from the formula.

$$\frac{\text{SNGFR}}{\text{filtration fraction}} = \text{Eq. 8}$$

As seen in Fig. 3, the length of the glomerular capillaries appears to differ under the two solutions. This is probably not due to a change in the functional length or a change in capillary permeability but rather to an error in determination of some of the parameters utilized in the calculations.

The results indicate that no equilibrium with respect to driving forces is obtained in the distal end of the glomerular capillary. Under normotensive conditions the net driving force is hypotension about 6 mm Hg. The mean net driving

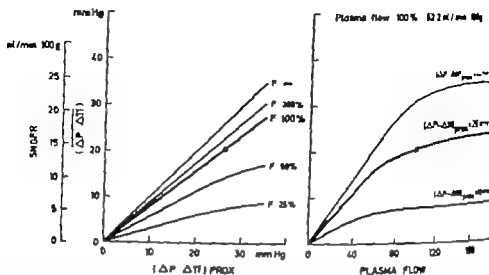


Fig. 4 The relation between the mean net driving force and the SNGFR and the driving force at the proximal end of the glomerulus (left panel) and of the single nephron plasma flow (right panel). The heavy lines represent normal conditions. The heavy lines represent normal flows and normal pressures respectively. The figure indicates that SNGFR is pressure-dependent whereas its dependency on flow is less pronounced. At a plasma flow of about half the normal, the plasma flow dependency is predominant.

force for glomerular filtration is estimated to be 20 mm Hg during normotension, decreased to 12 mm Hg when the perfusion pressure is reduced to 80 mm Hg. This reduction is proportionately somewhat greater than the reduction in single nephron GFR which decreases from 14.1 to 10.4 nl/min and 100 g b wt. The hydraulic permeabilities are calculated 0.7 and 0.8 nl/min 100 g mm Hg under the two conditions, respectively.

From the information on the kinetics of glomerular ultrafiltration under normal diuretic conditions the dependence of the filtration on pressure and on plasma flow is evaluated. For this purpose the permeability and functional length are assumed to be the same as under normal conditions.

Fig. 4 (left panel) shows the calculated relation between the net driving force at the proximal end of the glomerular capillaries, on the one hand, and the mean glomerular net driving force and the SNGFR, on the other. The different curves refer to different single nephron plasma flows. Analogously the right panel in the figure shows the relation between plasma flow on the one hand and the mean net driving force, and the SNGFR, on the other, for a number of assumed net driving forces in the proximal end of the glomerular capillaries. The heavy line represents the relation under normal pressure conditions. The black lines indicate normal antidiuretic conditions with respect to both plasma flow and net driving force. The calculations have been performed from Eq. 4.

The figure indicates that at normal plasma flows the SNGFR will be almost linearly related to the net driving force at the proximal end of the capillary. Moreover a moderate increase or decrease in plasma flow will cause relatively small changes in the SNGFR.

In Fig. 5 the influence of the pre- and postglomerular conductances on the mean net driving force and the SNGFR, and also their influence on the single nephron plasma

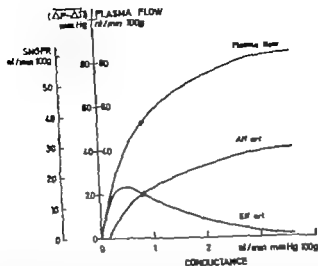


Fig. 5. The influence of pre- and postglomerular conductances on 1 the single nephron plasma flow (upper curve), 2 the SNGFR, and 3 the mean net driving force (lower curve). The black dots represent normal conditions. The conductance was changed in one vascular segment, while the other conductance was kept unchanged. A preglomerular relaxation will lead to an increased SNGFR. A postglomerular relaxation will lead to decreased SNGFR. A maximum value is obtained when the postglomerular conductance is about 1/2 of the control value. At very low postglomerular conductances the SNGFR and mean net driving force slowly decrease towards zero.

analysed (the black dots indicate normal conditions, under which the pre- and postglomerular conductances are approximately the same). This analysis involves calculation of the glomerular capillary pressure,  $P_{\text{Glc}}$  and the glomerular plasma flow  $F_{\text{Glc}}$ , as follows:

$$L_{\text{pre}}(P_{\text{pre}} - P_{\text{Glc}}) = L_{\text{post}}(P_{\text{Glc}} - 0) \quad \text{Eq. 9}$$

where  $L$  are the pre- and postglomerular conductances and  $P_{\text{pre}}$  the systemic blood pressure. The pressure in Bowman's space is assumed to be proportional to the SNGFR. This assumption would seem reasonable since an increased SNGFR will cause a tubular dilatation which will reduce the resistance for tubular fluid flow, but a concomitant decrease in fractional reabsorption will have the opposite effect (Lewy and Windhager 1968). It should be pointed out that in performing the analysis the preglomerular conductance was changed while the postglomerular conductance was kept at its normal value, and vice versa.

As expected the single glomerular plasma flow increased with an increase in either the pre- or the postglomerular conductance.

Relaxation of the preglomerular vessels (afr art. in Fig. 5), implying an increased conductance, will be concomitant with an increased SNGFR as a consequence both of an increased glomerular capillary pressure and of an increased glomerular plasma flow. Conversely a decreased conductance will cause a rapid decrease in SNGFR.

A successively increased conductance of the postglomerular vessels (eff. art.) will lead to a successively decreased SNGFR, whereas a decreased conductance will cause a rise in SNGFR.

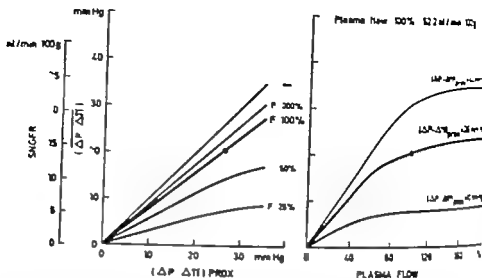


Fig. 4 The relation between the mean net driving force and the SNGFR and the driving force at the proximal end of the glomerulus (left panel) and of the single nephron plasma flow (right panel). The heavy lines represent normal flows and normal pressures respectively. The figure indicates that SNGFR is pressure-dependent whereas its dependency on flow is less pronounced. At a plasma flow of about half the normal, the plasma flow dependency is predominant.

force for glomerular filtration is estimated to be 20 mm Hg during normotension, down to 12 mm Hg when the perfusion pressure is reduced to 80 mm Hg. This reduction is proportionately somewhat greater than the reduction in single nephron GFR which decreases from 14.1 to 10.4 ml/min and 100 g b.wt. The hydraulic permeabilities are calculated to 0.7 and 0.8 ml/min/100 g mm Hg under the two conditions, respectively.

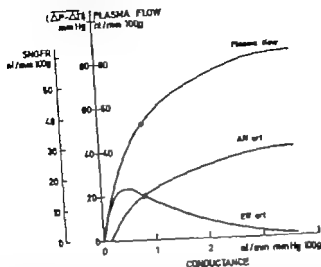
From the information on the kinetics of glomerular ultrafiltration under normal diuretic conditions the dependence of the filtration on pressure and on plasma flow is evaluated. For this purpose the permeability and functional length are assumed to be the same as under normal conditions.

Fig. 4 (left panel) shows the calculated relation between the net driving force at the proximal end of the glomerular capillaries, on the one hand, and the mean glomerular net driving force and the SNGFR on the other. The different curves refer to different single nephron plasma flows. Analogously, the right panel in the figure shows the relation between plasma flow on the one hand and the mean net driving force, and the SNGFR, on the other, for a number of assumed net driving forces in the proximal end of the glomerular capillaries. The heavy line represents the relation under normal pressure conditions. The black dots indicate normal antidiuretic conditions with respect to both plasma flow and net driving force. The calculations have been performed from Eq. 4.

The figure indicates that at normal plasma flows the SNGFR will be almost linearly related to the net driving force at the proximal end of the capillary. Moreover, a small increase or decrease in plasma flow will cause relatively small changes in the SNGFR.

In Fig. 5 the influence of the pre- and postglomerular conductances on the net driving force and the SNGFR, and also their influence on the single nephron plasma





The influence of pre- and postglomerular conductances on 1. the single nephron plasma flow (upper curve), 2. the SNGFR, and 3. the mean net driving force (lower curve). The black dots represent normal conditions. The conductance is changed in one vascular segment, while the other conductance was kept normal. A preglomerular relaxation will lead to an increased SNGFR. A postglomerular relaxation will lead to a decreased SNGFR. A maximum value is obtained when the postglomerular conductance is about half the control value. At very low postglomerular conductances the SNGFR and mean net driving force decrease towards zero.

analysed (the black dots indicate normal conditions, under which the pre- and postglomerular conductances are approximately the same). This analysis involves calculation of the glomerular capillary pressure,  $P_{\text{glom}}$ , and the glomerular plasma flow  $F_{\text{glom}}$ , as follows:

$$L_{\text{pre glom}}(P_{\text{sys}} - P_{\text{glom}}) = L_{\text{post glom}}(P_{\text{glom}} - 0) \quad \text{Eq. 9}$$

where  $L$  are the pre- and postglomerular conductances and  $P_{\text{sys}}$  the systemic blood pressure. The pressure in Bowman's space is assumed to be proportional to the SNGFR. This assumption would seem reasonable since an increased SNGFR will cause a tubular dilatation which will reduce the resistance for tubular fluid flow, but a concomitant decrease in fractional reabsorption will have the opposite effect (Lewy and Windhager 1968). It should be noted out that in performing the analysis the preglomerular conductance was changed while the postglomerular conductance was kept at its normal value, and vice versa.

As expected the single glomerular plasma flow increased with an increase in either the pre- or the postglomerular conductance.

Relaxation of the preglomerular vessels (aff. art. in Fig. 5), implying an increased conductance, will be concomitant with an increased SNGFR as a consequence both of an increased glomerular capillary pressure and of an increased glomerular plasma flow. Conversely a decreased conductance will cause a rapid decrease in SNGFR.

A successively increased conductance of the postglomerular vessels (eff. art.) will lead to a successively decreased SNGFR, whereas a decreased conductance will cause a rise in SNGFR.

which will reach its maximum when the conductance has decreased to about half of the normal value. A further reduction will cause a rapid fall in SNGFR. This fall is then due to a concomitant decrease in single glomerular plasma flow.

### Discussion

The ideas obtained in this study about the kinetics of glomerular filtration contrast sharply with those obtained by Brenner *et al.* (1971) in investigations on the unique mutant *le* rat strain. In their studies the driving force in the proximal end of the glomerulus was about 10 mm Hg. Along the glomerular capillaries the driving force was found to decrease successively, reaching a value close to zero somewhere at the distal end. Owing to the uncertainty as to where the driving force was zero, the filtration permeability coefficient could not be determined. On the basis of their findings it is clearly evident that the glomerular filtration rate is to be highly dependent on plasma flow—an interpretation which is supported by the present evaluation seen in Fig. 4 (the plasma flows in the Wistar strain are less than half of those obtained in the present study). The hydrostatic pressure within the glomerulus will nevertheless be a determinant of the filtration rate in accordance with that predicted in Fig. 4. Its influence will, however, be fairly moderate since the oncotic pressure shows a steep increase (as compared with the low hydrostatic pressure) when the filtration fraction increases to the figure of 0.3 found in the Wistar strain (see Fig. 7).

Further, according to the view on the dynamics of glomerular ultrafiltration presented by Brenner and coworkers the glomerular filtration rate can only be increased by a reduction of the preglomerular vessels, as the latter will cause an increase in both the glomerular capillary pressure and the plasma flow (Fig. 4). An increase in the postglomerular resistance will not cause an increase in filtration rate, since the effect of a higher hydrostatic pressure within the glomerulus will be counteracted by the decreased plasma flow.

The discrepancy between the evaluation made in the present study and that made by Brenner *et al.* is thus not only quantitative but also qualitative. This is explained by differences in the values for both glomerular plasma flow (52 versus 20 nl/min/100 g) and glomerular capillary hydrostatic pressure (63 versus 45 mm Hg). The most interesting finding in the present investigation is that a filtration equilibrium is not attained, and that the filtration rate can thus be controlled by both the pre- and postglomerular resistances and can vary with changes in plasma flow. This means that the glomerular filtration rate can remain constant despite changes in plasma flow, as has been demonstrated experimentally (Kilgus *et al.* 1968 and Pitts 1968).

If, however, the glomerular plasma flow in the mutant Wistar rat is increased to about 50 nl/min/100 g—i.e. about the same value as presented in this paper (by iso-oncotic plasma expansion)—a state of filtration non-equilibrium will be achieved (Deen *et al.* 1973). In this case the mean net driving force could be calculated to be about 4–6 mm Hg, thus considerably lower than that reported here. The hydraulic permeability will accordingly be estimated to be about 0.08 nl/s/mm Hg, which would mean some 1.6 nl/min and 100 g b.w.t./i.e. about twice the figure obtained in the present study.

Using the Poiseuille formula and assuming a pore radius of 50 Å and a viscosity of

filtrate of 0.01 poise (Landis and Pappenheimer 1963), the permeability factor  $A_p/\Delta x$  (area over pore length per glomerulus) is calculated as 3 cm/100 g rat, thus in a 300 g rat, it is 9 cm.

This determination will not permit calculation of the pore area,  $A_p$ . Assuming a pore size of 0.3  $\mu$ m, however, the area per glomerulus of a 300 g rat will be  $2.71 \cdot 10^{-4}$  cm<sup>2</sup> and a total surface area of  $1.9 \cdot 10^{-4}$  cm<sup>2</sup> as found by Kirkman and Stowell (1942), the total pore area is estimated to be 14%. In the dog kidney Gasson *et al.* (1974) estimated the factor  $A_p/\Delta x$ , by the sieving of fractions of polyvinylpyrrolidone, to be  $1.35 \cdot 10^4$  cm per kidney. If the corresponding factor of 9 cm for one glomerulus, found here is multiplied by 30 000, i.e. the number of glomeruli in a rat kidney and assuming that the in vivo kidney weight is about 1.7 g, the permeability per gram kidney is calculated to be  $1.6 \cdot 10^4$  cm. This is in reasonable accordance with that of Gasson *et al.* These  $A_p/\Delta x$  values are considerably lower than those in capillary beds of other organs, but are similar to those obtained for tubular capillaries. The driving force for the fluid reabsorption has been estimated in the department to be about 14 mm Hg (Wunderlich *et al.* 1971 Wolgast *et al.* 1973 Källskog and Wolgast 1973). The more precise calculation of the present study gives a net driving force of 11 mm Hg, i.e. about half the driving force valid for glomerular ultrafiltration. As transmembrane fluid flow is about the same as over the glomerular membrane, the permeability factor  $A_p/\Delta x$ , per gram kidney weight will be some  $3 \cdot 10^4$  cm. The total area of the peritubular capillaries is, however, considerably larger, i.e. about 350 cm<sup>2</sup> per gram kidney (Kupfjerg and Brauer 1962). Assuming the same pore length of 0.3  $\mu$ m as for glomerular capillaries (the pore length is probably less than that in the glomerular membrane the latter will consist of both the capillary and tubular wall), the fractional pore area will be about 2.

### References

- B. A. and A. N. RICHARDS, Passage of protein molecules through glomerular membranes. *J. Biol. Chem.* 1941, 141, 791.
- CHURCH, B. M., J. L. TROY and T. M. DAUMARTY, The dynamics of glomerular ultrafiltration in the rat. *Am. J. Physiol.* 1971, 220, 1776-1780.
- CHURCH, B. M., W. M. DURN and C. R. ROBERTSON, *The physiological basis of glomerular ultrafiltration in laboratory animals and man*. Ed. U. Thirum, London 1974, 336.
- CHURCH, B. M., P. P. DECAUDOT, A. VERNIER and P. P. LAMBERT, Autoregulation of effective glomerular filtration pressure. *Amer. J. Physiol.* 1974, 226, 616-623.
- CHURCH, B. M., J. A. MANDOOK, G. BEAL and H. D. PAGEL, Pressure in the glomerular capillaries of the rat kidney and its relation to arterial blood pressure. *Pflügers Arch. ges. Physiol.* 1966, 228, 369-374.
- CHURCH, B. M. and M. W. ROSE, Driving forces over the peritubular capillary membrane in the rat kidney during micropuncture and tubule expansion. *Acta physiol. scand.* 1973, 89, 116-125.
- CHURCH, B. M., T. O. LINDQVIST, H. R. LINDQVIST and M. WOLGAST, Kinetics of the glomerular ultrafiltration in the rat kidney. An experimental study. *Acta physiol. scand.* 1973, 1, press.
- CHURCH, B. M., J. KALLSKOG and E. LÖNNBERG, Role of autoregulation in maintaining glomerular filtration rate at large urine flow. *Acta physiol. scand.* 1969, 76, 4-39.
- CHURCH, B. M. and R. E. S. CHURCH, Renal filtration surface in the albino rat. *Am. J. Anat.* 1942, 82, 373-384.
- CHURCH, B. M. and B. BRAUER, Quantitative Untersuchungen über kapillare und Tubuli der Nierenrinne. *Z. Zellforsch.* 1962, 514, 766-778.

- LAMBERT P. P., A. VERNIORY, J. P. GASSÉ and P. FICHERROUX, Sieving equations and effective filtration pressure. *Kidney Intern.* 1972, 2: 131-146.
- LANDIS, E. M. and J. R. PAPPENHEIMER, Exchange of substances through the capillary. *in: Basic physiology Circulation II* Waverly Press, USA, 1963, 961-1034.
- LEWY J. E. and E. E. WINDMAGER, Peritubular control of proximal fluid reabsorption in the rat. *Amer J Physiol* 1968 214: 943-954.
- PAPPENHEIMER, J. R., E. M. RENKIN and L. BORRERO, Filtration diffusion and molecular sieving in peripheral capillary membranes. A contribution to the pore theory of capillary permeability. *in Physiol.* 1951 167: 13-46.
- PITTS, R. F. *Physiology of the kidney and body fluids*. Chicago, Year Book Medical Publ. 1962 III.
- WALLINER, G. Renal clearance of dextran as a measure of glomerular permeability. *Acta Læxologica* 1954, Suppl. 4.
- SCHIRMANN J. Physical forces and transtubular movement of solutes and water. *in Kidney and direct physiology* Physiology series I vol. 6, Ed. K. Thurau, Butterworths, London, 1974 157-179.
- SOLOMON A. K. Characterization of biological membranes by equivalent pores. *J. gen. Physiol.* 1968 51: 335-364.
- VERNIORY A., R. DU BOIS, P. DECOUDT, J. P. GASSÉ and P. P. LAMBERT The measurement of the permeability of biological membranes. Application to the glomerular wall. *J. Gen. Physiol.* 1973 62: 14.
- WOLGAST M., E. PERSSON, J. SCHIRMANN, H. ULFENDAHL and P. WUNDERLICH, Colloid osmotic pressure of the subcapsular interstitial fluid of rat kidneys during hydropenia and volume expansion. *Z. Arch. ges. Ph. med.* 1973, 340: 123-131.
- WUNDERLICH, P., E. PERSSON, J. SCHIRMANN, H. ULFENDAHL and M. WOLGAST Hydrostatic pressure in the subcapsular interstitial space of rat and dog kidneys. *Pflügers Arch. ges. Physiol.* 1971 326: 36.

## The Effects of Hypothermia on Submaximal and Maximal Work Performance

By

MERVYN DAVIES, BJÖRN EKLÖM, ULF BERGH and INGE-LEI KANSTRUP-JENSEN

**Keywords:** Hypothermia, exercise, heart rate, atropine,  $\beta$  blockade, aerobic power output

It is known that hypothermia affects physical performance (Keatinge 1969) and may be fatal if exposure is severe (Pugh 1964). It has also been shown that swimming in water ( $18^{\circ}\text{C}$ ) results in an increased metabolic rate at a given speed and a decreased rate at a given oxygen uptake (Nielsen 1973, Nadel *et al.* 1974, Holmér and Bergh 1974). Similar results have been obtained during submaximal bicycle exercise (Nadel *et al.* 1974), but to our knowledge no attempts have been made to explain this HR response to cold. We also intended to study the effect of hypothermia on the response to maximal exercise under standardized laboratory conditions.

In the present study we made 5 normal healthy male subjects hypothermic by allowing them to swim at a speed of 0.55 m/s ( $V_{O_2}$  about 50% of  $V_{O_{2\max}}$ ) for 30 min (until rectal temperature of approximately  $35^{\circ}\text{C}$  was reached) in a swimming flume (Åstrand and Engle-1972) containing water at  $18^{\circ}\text{C}$ . Immediately after the end of the swim the response to submaximal and maximal exercise on an arm and leg bicycle ergometer under standardized laboratory conditions with and without atropine (2 mg i.v.) was measured and the data collected were compared to results obtained during normal cycling. In addition one subject was also studied after  $\beta$ -blockade with propranolol (10 mg i.v.).

The results show that following hypothermia  $V_{O_2$  and  $V_{O_{2\max}}$  at a given submaximal work rate of 100 W increased ( $p < 0.05$ , Table 1) whereas the heart rate was reduced by 14 beats/min. Atropine reversed this latter change but was without effect on the observed increase in the metabolic cost of work. The effect of atropine on the heart rate was of the same order of magnitude as reported earlier from studies in normothermic conditions (Eklöm *et al.* 1974). In contrast the administration of propranolol did not further reduce HR during submaximal exercise in hypothermia. At maximal effort hypothermia significantly reduced  $V_{O_{2\max}}$ ,  $V_{O_{2\max}}$  and HR max by 2.5 l/min, 0.42 l/min and 26 beats/min respectively. These values were unaffected by atropine (Table 1). These data show that hypothermia has a profound effect on physical performance by reducing maximal aerobic power whilst increasing the submaximal cost, placing greater relative strain on the individual. The change in  $V_{O_{2\max}}$  was related to the decrease in cardiac frequency and this relationship was un-

TABLE I

	$V_E$ BTPS l/min	$V_{O_2}$ STPD l/min	$V_{CO_2}$ STPD l/min	RQ	EE kcal
(a) Submaximal responses to exercise at a given work load of 100 W					
Normothermia n=5	41.1 ± 7.1	1.52 ± 0.09	1.43 ± 0.18	0.94 ± 0.14	14.3
Hypothermia n=5	64.1 ± 17.8	1.90 ± 0.21	1.79 ± 0.29	1.00 ± 0.09	19.7
Hypothermia (plus Atropine) n=5	64.5 ± 15.5	1.90 ± 0.18	1.97 ± 0.30	1.04 ± 0.11	19.9
(b) Maximal responses to exercise					
Normothermia n=5	165.0 ± 9.5	3.87 ± 0.30	4.40 ± 0.32	1.13 ± 0.02	14.1
Hypothermia n=5	145.3 ± 16.4	3.45 ± 0.33	4.20 ± 0.35	1.22 ± 0.12	13.8
Hypothermia (plus Atropine) n=5	140.2 ± 70.9	3.44 ± 0.38	3.99 ± 0.41	1.16 ± 0.04	13.1
Significance Normothermia-hypothermia	P < 0.001		P < 0.01	P < 0.05	
Hypothermia-hypothermia (plus Atropine)	++P < 0.001		++P < 0.01	+P < 0.05	

affected by atropine. The results from this study suggest that hypothermia may be direct effect on the cardiac muscle and possibly that the  $\beta$ -receptor activity is reduced in hypothermia.

### References

- ÅSTRAND, P.-O. and RODAHL, A. swimming Papa. *J appl Physiol* 1972 33 514.  
 EKBLOM, B., A. GOLDBERG, Å. KILBOM and P.-O. ÅSTRAND. Effects of atropine and propranolol on oxygen transport system during exercise in man. *Scand J clin Lab Invest* 1972 36 13-4.  
 HOLMÉR, I. and U. BERGH. Metabolic and thermal response to swimming in water at varying temperature. *J appl Physiol* 1974 37 702-705.  
 KEATINGE, W. R. *Swimming in cold water* 1969 Oxford, England. Blackwell.  
 NADAI, E. R., I. HOLMÉR, U. BERGH, P.-O. ÅSTRAND and J. A. J. STOLWIJK. Energy expenditure during man. *J appl Physiol* 1974 36 465-471.  
 NIELSEN, B. Metabolic reactions to cold during swimming at different speeds. *Arch Sci Phys Med* 1977 27 207-211.  
 PUGH, L. G. C. Deaths from exposure on Four 1 ms Walking Competition, March 14-15, 1964. *BMJ* 1964 1210-11.

## Capillary Density in Skeletal Muscle of Man

By

PER ANDERSEN

the basic work on skeletal muscle capillaries by Krogh (1918-19), the capillary density in human skeletal muscle has been described both with respect to red and white muscles and aging. Similar studies on man are very few due to methodological limitations. In the present study a new method has been used making it possible to describe capillary density and number of capillaries around different fibre types in human skeletal muscle.

In biopsy samples from the lateral portion of the quadriceps femoris muscle were taken from 9 healthy subjects. 5 subjects were untrained (group A,  $\dot{V}O_{2\max}$  44 ml/kg min (41-53)), and 4 subjects were endurance-trained (group B,  $\dot{V}O_{2\max}$  63 ml/kg min (58-73)). Three of the endurance-trained subjects (group C) completed a training program 4 times a week for about 7 weeks, pedalling a bicycle ergometer for 20 min at intensity requiring approximately 80% of  $\dot{V}O_{2\max}$ . This resulted in a 13% (9-20%) increase in  $\dot{V}O_{2\max}$ .

Muscle samples were isolated in an embedding medium and frozen in isopentane cooled to liquid nitrogen. Serial transverse sections (10  $\mu$ m thick) were cut in cryostat at -20°C. For fibre classification type I, IIA, IIB, and IIC sections were stained for myofibrillar ATPase, preincubated at various pH (Gale and Kaiser 1970). Sections to be stained for capillaries were kept at -20°C.

Staining procedure: Bring sections to room temperature; fix in Carnoy's fixative (10 min, 21°C); rinse in distilled water; digest glycogen with 1%  $\alpha$ -amylase (Sigma A-6753; 30 min, 37°C); rinse in distilled water; oxidize in 1% periodic acid ( $HIO_4$ ; 10 min, 21°C); stain with Schiff's reagent; rinse in water; dehydrate, clear and mount. This method (Amylose-PAS) stains most likely macropolyisocyanides in the muscle and capillary basement membranes (Plate I). The staining was photographed and copies produced by microcopy. Fibre types were classified on the photograph and capillaries were confirmed by direct microscopy. Fibre types were plotted on the photograph. Fibre area was measured by the grid method (Ludroff and Terjorg 1964, 69). The number of fibres of each type used for measuring area was based on the fibre composition within A (mean and range): type I 38 (20-60), type IIA: 27 (9-58), type IIB 14 (3-22) and type IIC 3 (1-4). The area data are not accepted unless the total area calculated as mean area and number of each fibre type within A agreed to within  $\pm 5\%$  of the actual measured area.

Because of the low number of type IIC fibres, these fibres were not included in the statistical analysis. The mean values, SE and range for the whole group (n = 9) are given in Table I. Two subjects in group B had no type IIB fibres (n = 7).

The mean capillary density of  $317 \pm 15$  cap./mm<sup>2</sup> corresponds well with the electron-microscope study (Saltin *et al.* 1968), but is lower than light-microscope data (Hermanssen and Wachtlov 1971). The number of capillaries around type I fibres was not different from type IIA fibres, both were higher than the number around type IIB fibres (p < 0.01). The fibre areas (type I  $4980 \pm 350$  ( $\mu$ m)<sup>2</sup>; type II  $5500 \pm 330$  ( $\mu$ m)<sup>2</sup>) are within the range reported in the literature (see Gollnick *et al.* 1972). Type IIA area was greater than either type I area or type IIB area (p < 0.01). There was no difference between mean fibre area of type I and IIB.

TABLE I. Mean values, SE and range for measured and calculated muscle characteristics a 1-r, n=6).

	$\dot{V}O_2$ max ml/kg min	A mm <sup>2</sup>	Fibre composition in area A					Capillaries per mm <sup>2</sup>	Capillaries per fibre
			I	% IIA	% IIB	% IIC	No. of fibre		
Mean	34	0.593	59	79	11	1.5	113	317	1.6
S.E.	4	0.090	6	4	2	0.7	14	15	0.17
Range	41	0.284	29	15	0	0	61	299	0.95
	75	1.116	82	58	21	6.3	180	390	2.0

When the mean number of capillaries around a fibre type was expressed relative to mean area for that fibre type, no difference was found between type IIA and IIB, but were lower ( $p < 0.01$ ) than type I. These data are reciprocally proportional to the diffusion distance described by Krogh (1918/1919) and are compatible with the biologic and contractile differences between the type I and II fibres (Easén *et al.* 1975). However, the data did not reflect the biochemical differences between type IIA and IIB fibres. Type I fibres have more capillaries than type II fibres, capillary density varies with composition (group A type I 45%  $\pm$  8 of total area, 48%  $\pm$  8 of total number 50 cap./mm group B type I 71%  $\pm$  5 of total area, 72%  $\pm$  4 of total number 355/170 mm (group A vs. B  $p < 0.01$ )).

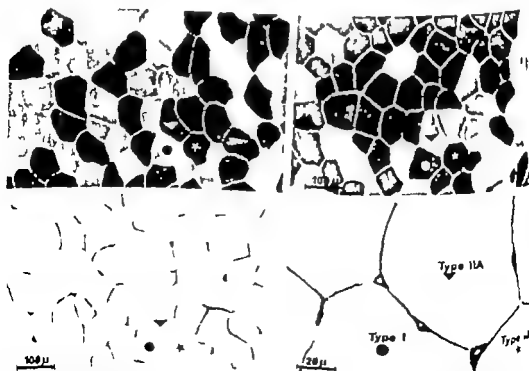


Plate 1 Photomicrographs of serial sections. Upper left and right ATPase staining, preincubated at 10.3 and 4.6, respectively (86X). Lower left and right Amylase-Pa staining (86 and 430X).



of capillaries type			Fibre area ( $\mu\text{m}^2$ ) type				Number of capillaries around fibre type per fibre type area $10^{-4} (\mu\text{m})^{-2}$			
IIA	IIB	IIC*	I	IIA	IIB	IIC*	I	IIA	IIB	IIC*
4.5	3.5	4.5	4 980	5 770	4 570	3 900	1.00	0.79	0.74	1.26
0.1	0.4	0.7	150	340	460	540	0.03	0.02	0.03	0.25
3.0	2.0	3.0	3 320	3 470	2 960	1 640	0.90	0.71	0.63	0.77
5.8	3.2	7.0	6 200	6 780	4 360	3 260	1.34	0.84	0.84	2.44

group C fibre composition was the same before and after training (type I respectively 13 and 31%  $\pm 7$  of total area), but the capillary density increased with training from 1.5 cap/mm to 3.56  $\pm 23$  cap/mm ( $p < 0.05$ ). The capillary/fibre ratio varied also with the training state (group A: 1.30  $\pm 0.13$  group B: 2.15  $\pm 0.12$  ( $p < 0.01$ ); group C before and after training: 1.39  $\pm 0.18$  and 1.95  $\pm 0.06$  ( $p < 0.05$ )). The difference was not only due to a different mean fibre area (group A: 4 500  $\pm 374$  ( $\mu\text{m}^2$ ); group B: 6 050  $\pm 60$  ( $\mu\text{m}^2$ ); group C: no significant change with training) (Hermansen and Wachtlova 1971), but also the difference in fibre composition and capillary density did affect the value.

Study is supported by the Danish National Science Research Council.

### References

- ALLEN, M. H. and K. K. KARMAN, Three "oxygen ATPase" systems: The nature of their pH lability and (pH) dependence. *J. Histochem. Cytochem.* 1970, 18, 670-672.
- BRADY, L. and K. TOLLBOELLER, Area estimates of transversely sectioned muscle fibres. *Z. Anat. Med.* 18 69, 161-178.
- CHAMBERLAIN, J. J., HERMANSSEN, A. W. T. and B. SALTIN, Metabolic characteristics of fibre types in human skeletal muscle. *Acta physiol. scand.* 1975, 95, 153-163.
- LUICK, P. D., R. B. ARMSTRONG, C. W. SALMON, IV, K. PRINZ and B. SALTIN, Enzyme activity and fiber properties in skeletal muscle of untrained and trained man. *J. appl. Physiol.* 1972, 33, 31-319.
- HERMANSEN, L. and M. WACHTLOVA, Capillary density of skeletal muscle in well-trained and untrained man. *J. appl. Physiol.* 1971, 32, 860-863.
- KIL, A., The number and distribution of capillaries in muscles with calculations of the oxygen pressure and necessary for supplying the tissues. *J. Physiol. (Lond.)* 1918, 1919, 52, 409-415.
- DE, B. B. BLUMBERG, J. H. MITCHELL, R. L. JOHNSON, J. K. WILBERTHAL, and C. B. CHAPMAN, Lysine - exercise after bed rest and training. *Circulation* 1968, Suppl. 7.

TABLE I Mean values, SE and range for measured and calculated muscle characteristics,  $n = 6$ 

	$\dot{V}O_2$ max ml/kg min	A mm <sup>2</sup>	Fibre composition in area A				Capillaries per mm <sup>2</sup>	Cap- per litre
			I	% IIA	IIB	% IIC		
Mean	34	0.593	59	29	11	1.5	113	1.0
S.E.	4	0.090	6	4		0.7	14	0.1
Range	41	0.44	79	15	0	0	61	0.5
	75	1.116	8	58	1	6.3	180	0

When the mean number of capillaries around a fibre type was expressed relative mean area for that fibre type, no difference was found between type IIA and IIB, but were lower ( $p < 0.01$ ) than type I. These data are reciprocally proportional to the  $\dot{V}O_2$ -diffusion distance described by Krogh (1918/1919) and are compatible with the haemodynamic and contractile differences between the type I and II fibres (Essén *et al.* 1975). However, the data did not reflect the biochemical differences between type IIA and IIB fibres. Since type I fibres have more capillaries than type II fibres, capillary density varies with composition (group A: type I 45%  $\pm$  8 of total area, 48%  $\pm$  8 of total number of cap/mm; group B: type I 71%  $\pm$  5 of total area, 72%  $\pm$  4 of total number 355/17 mm (group A vs. B  $p < 0.01$ )).

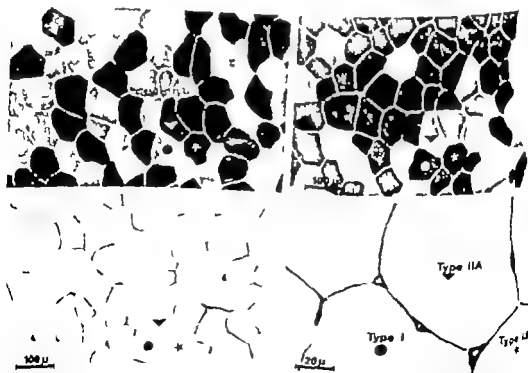


Plate I. Photomicrographs of aerial sections. Upper left and right: ATPase staining, preincubated at 10.3 and 4.6, respectively (84X). Lower left and right: Amylase PAS staining (36X and 430X).



Fig. 1 Individual CBF and CMRO<sub>2</sub> values in rats under nitrous oxide anaesthesia (N<sub>2</sub>O) and in unanaesthetized but air-saturated animals (N<sub>2</sub>) without or with previous adrenalectomy. Mean values are indicated by horizontal bars.

1 a CBF just above 100 ml (100 g)<sup>-1</sup> min<sup>-1</sup> (data from Hägerdal *et al.* 1975). When the flow from occlude supply was withdrawn CMRO<sub>2</sub> almost doubled with a corresponding increase in CBF. This increase in CMRO<sub>2</sub> was almost completely prevented by previous adrenalectomy. Furthermore, since it could also be prevented by a beta blocker (propranolol), we conclude that the increase in CMRO<sub>2</sub> represented a stress response mediated by adrenaline. Although it has been accepted as a central dogma that CMRO<sub>2</sub> remains constant in a variety of functional states, Kety (1950) has previously reported an increase in CMRO<sub>2</sub> in a patient who showed signs of severe anxiety and King *et al.* (1952) found that adminis-

## Increase in Cerebral Oxygen Uptake and Blood Flow in Immobilization Stress

By

CHRISTER CARLSSON, MAGNUS HÄGERDAL and Bo K. Sævi

In the course of a study of cerebral metabolism and circulation in hypoxia we are presently examining the modulating influences of various drugs and anesthetics. Since at least one of these drugs (diazepam) appeared to interact with the anesthetic used (nitrous oxide) it was deemed necessary to study the effect of nitrous oxide by carrying out measurements of cerebral blood flow (CBF) and oxygen consumption ( $CMR_{O_2}$ ) in unanesthetized but paralyzed rats. The results of these studies revealed that in the absence of anesthesia, immobilization is accompanied by a pronounced rise in  $CMR_{O_2}$  and CBF which appears not to be mediated by adrenaline. The present article gives a preliminary account of the findings.

Male Wistar rats were anesthetized with 2-3% halothane, tracheotomized, intubated by tubocurarine chloride and maintained ventilated during surgery on 70%  $N_2O$  and 30%  $O_2$ . The respirator was adjusted to give a  $P_{aO_2}$  of 35-40 mm Hg, and body temperature kept close to 37°C. The femoral arteries were cannulated for blood pressure recording and for anaerobic sampling of arterial blood, and the caudal part of the superior sagittal sinus was exposed by a burr hole for sampling of cerebral venous blood. CBF was determined with a  $^{133}Xe$  modification of the Kety and Schmidt (1948) technique, and arterial cerebral venous oxygen content was measured polarographically (Norberg and Sævi 1971).  $CMR_{O_2}$  was calculated as the product of the CBF and the arteriovenous difference in  $O_2$  content.

Two groups of unanesthetized animals were studied. In both, operative procedures were carried out under anesthesia as described above. In one group, bilateral adrenalectomy was carried out by a dorsal approach. In the other, no adrenalectomy was performed. In both groups, operative incisions were carefully sutured and the wounds were infiltrated with 0.5% Carbocain (Bofors, Sweden). The animals were then removed from the head-holder and allowed to rest in a bed of cotton wool. Care was taken to exclude or minimize all sound stimuli. After that the animals had been undisturbed for about 10 min, the  $N_2O$  supply was discontinued and the inlet of the respirator was connected to a bag containing  $^{133}Xe$  in 70%  $N_2$  and 30%  $O_2$ . After a saturation period of 15 min repeated samples were taken from artery and vein for measurements of CBF and  $CMR_{O_2}$ . Great care was taken during sampling not to disturb the animals.

As seen in the figure, animals under nitrous oxide anesthesia had a  $CMR_{O_2}$  of 1

## Reduced Urinary Noradrenaline Excretion during Rest, Exercise and Cold Stress in Trained Rats: a Comparison between Physically Trained Rats, Cold-Acclimated Rats and Warm-Acclimated Rats

By

INGEGERD ÖRTMAN and NILS O. SJÖSTRAND

Received 21 February 1975

### Abstract

ÖRTMAN, I. and N. O. SJÖSTRAND. *Reduced urinary noradrenaline excretion during rest, exercise and cold stress in trained rats: comparison between physically-trained rats, cold-acclimated rats and warm-acclimated rats.* Acta physiol. scand. 1975. 95. 209-218.

Physically trained rats were compared with cold-acclimated rats. Trained as well as cold-acclimated rats had cardiac and adrenal hypertrophy. Cardiac noradrenaline (NA) content was increased in both groups of rats but only the trained rats had an increased cardiac NA concentration. The adrenal NA content was increased in both groups but only the trained rats had an increased adrenal content of adrenaline (A). The spleen of trained rats had an increased NA content, while that of cold-acclimated rats had reduced NA content. The submandibular glands of cold-acclimated rats were enlarged and had an increased NA content. Trained as well as cold-acclimated rats had lower urinary NA excretions during rest, exercise and during cold stress than compared with controls. However, only the trained rats had a reduced urinary NA excretion after exercise, whereas there was no difference between the increases of cold-acclimated and control rats. Six months after cessation of training, ex-trained rats still had an elevated heart ratio and reduced urinary NA excretion after exercise. It is suggested that physical training gives cross tolerance to cold stress, while cold-acclimation does not lead to "cross tolerance" to exercise.

In a previous study (Örtman, Sjöstrand and Swedin 1972) it was found that, after a period of exercise, trained (chronically exercised) rats display a considerably smaller increase in urinary catecholamine excretion than do untrained rats. Together with the finding of a lower rate of cardiac noradrenaline (NA) turnover in trained rats than in controls both in rest and during exercise, this was taken as an indication of an adaptation of the sympatho-adrenal system to chronic exercise, manifested as a better transmitter economy in the trained animal (Örtman *et al.* 1972).

In the sense of reduced demands for catecholamines (CA) in order to maintain certain functions, adaptation of the peripheral adrenergic system is well known as a characteristic of cold acclimation in the rat. Thus, cold-acclimated rats exhibit an increased sensitivity

tration of adrenaline to human subjects gave small but significant increases in CBF and  $CMR_{O_2}$ . The present results, which are in line with these previous observations, demonstrate that stressful situations may cause a dramatic increase in cerebral metabolism.

We want to acknowledge the excellent technical assistance given by Miss Gunilla Olsson. The work was supported by grants from the Swedish Medical Research Council (Project No. 14 X 263 and 411) and from U.S. PHS No 5 R01 NS 07834-06 from N I H.

### References

- HÄGERDAL, M., J. HARP, L. NILSSON and B. K. SUND, The effect of induced hypothermia upon oxygen consumption in the rat brain. *J. Neurochem.* 1975. **24**: 311-316.
- KETY, S. S. and C. F. SCHMIDT, The nitrous oxide method for the quantitative determination of blood flow in man: theory, procedure and normal values. *J. Clin. Invest.* 1948. **27**: 476-481.
- KETY, S. S., Circulation and metabolism of the human brain in health and disease. *Amer. J. Med.* **8**: 205-217.
- KING, B. D., L. SOKOLOFF and R. L. WEISSLER, The effects of  $\beta$ -epinephrine and  $\beta$ -nor-epinephrine on cerebral circulation and metabolism in man. *J. clin. Invest.* 1952. **31**: 273-279.
- NORBERG, K. and B. K. SUND, Quantitative measurement of blood flow and oxygen consumption in rat brain. *Acta physiol. scand.* 1974. **91**: 154-164.

aged, titrated and re-centrifuged (see Swobbe 1971), whereafter the extracts were oxidized. One tenth of 0.07 M EDTA was added to the assay samples whereafter the CA were adsorbed on alumina and as described by Swobbe (1971). CA were produced and determined fluorimetrically according to Chang (1966). The CA values are expressed as  $\mu\text{g}$  free base.

#### Statistical procedures

Means and standard error of the means (S.E.) were computed according to ordinary formulae. The values were tested and compared by the Student *t*-test. A *p*-value of 0.05 or less was considered significant.

## Results

### Body and organ weights (see Table I and III)

In our earlier studies (Östman and Sjöstrand 1971, Östman *et al.* 1972) the trained rats had significantly lower body weight (b.w.) than the controls, while the body length remained unchanged, and significantly greater heart weights, heart ratios and adrenal weights, thus a rat exhibited the criteria of successful physical training. The cold-acclimated rats also had evidence of cardiac hypertrophy with significantly greater heart weight and heart ratio, and of significantly greater adrenal weight, but their final b.w. did not differ significantly from the controls and neither did their body lengths. However the b.w. of the cold-acclimated rats did not differ significantly from that of the trained rat either. Furthermore, weight of the submandibular salivary glands was significantly greater in the cold-acclimated rats than in controls and trained rats.

Six months after cessation of the swimming training the only difference that remained between the controls and the ex-trained rats was that the ex-trained rats still had a highly significantly greater heart ratio (Table III).

Table I. Effects of physical training and cold-acclimation on body and organ weights in the rat (Mean S.E.).

	Initial b.w. (g)	Final b.w. (g)	Final body length (cm)	Heart weight (g)	Heart ratio (g/100 g b.w.)	Salivary glands (g)	Spleen weight (g)	Adrenal weight (mg)
Control rats (2)	180 $\pm$ 2	440 $\pm$ 7	23.4 $\pm$ 0.1	1.23 $\pm$ 0.02	0.275 $\pm$ 0.003	0.64 $\pm$ 0.02	0.78 $\pm$ 0.03	40 $\pm$ 1
Trained rats (1)	182 $\pm$ 2	408 $\pm$ 7	23.4 $\pm$ 0.2	1.32 $\pm$ 0.02	0.325 $\pm$ 0.003	0.65 $\pm$ 0.01	0.72 $\pm$ 0.01	43 $\pm$ 1
Cold-acclimated rats (n = 11)	183 $\pm$ 2	427 $\pm$ 5	23.1 $\pm$ 0.2	1.32 $\pm$ 0.02	0.315 $\pm$ 0.004	0.72 $\pm$ 0.01	0.79 $\pm$ 0.02	45 $\pm$ 2
Test of significance by Student's <i>t</i> -test								
control trained	n.s.	<i>p</i> 0.01	n.s.	<i>p</i> 0.01	<i>p</i> < 0.001	n.s.	n.s.	<i>p</i> 0.02
control cold-accl.	n.s.		n.s.	<i>p</i> 0.01	<i>p</i> 0.001	<i>p</i> 0.01	n.s.	<i>p</i> < 0.01
trained cold-accl.	n.s.	n.s.	n.s.	n.s.	n.s.	<i>p</i> 0.01	<i>p</i> 0.01	n.s.

n.s. not significant.

to the calorogenic effect of CA (Halek and Carlson 1957 Depocas 1960, Heron 1961) when exposed to a cold environment they excrete less NA than do warm-acclimated (Leduc 1961). However exposure to cold also involves increased demands on the cardiovascular system of the rat. The cardiac output increases (Janaky and Hart 1968) and acclimated rats develop cardiac hypertrophy (Emery *et al.* 1940).

Thus, there are apparent similarities between the cold-acclimated rat and the more. Both have a functional hypertrophy of the heart and both display a reduced response to the adrenergic transmitter when exposed to an arduous situation to which the organism is adapted, i.e. cold and exercise, respectively. This has prompted us to make a comparison between cold-acclimated and trained rats. The present report deals with the urinary excretion of trained rats, cold-acclimated rats and sedentary warm-acclimated rats subjected to cold stress or to exercise, as well as during rest at room temperature. In addition the NA levels in the heart and the amounts of adrenaline (A) and NA in the adrenal glands have been measured. Furthermore, the effect of cessation of training on urinary CA excretion and organ CA levels has been investigated.

### Material and Methods

Male Sprague-Dawley rats initially weighing about 180 g and being 40–45 days old were randomly divided into 5 groups. Two groups of rats were submitted to daily exercise during 14 weeks and one group was acclimated to cold during this period. The remaining two groups served as controls. The control animals, the trained rats were kept at a room temperature of about 24°C. The cold acclimated rats were kept at a temperature of 4°C. All rats were housed in groups of 3–6 animals and received food and tap water *ad libitum*. All cages were furnished with sawdust as bedding. The illumination essentially follows day-light.

The rats were exercised by swimming in baths, 100 × 70 × 60 cm, with a water depth of 40 cm. 12–15 rats were swimming simultaneously in the same bath. The water was kept at a temperature of 15 ± 1°C. The period of swimming was initially 1 h and was gradually increased to 2 h per day as described previously (Östman *et al.* 1972).

One group of trained rats, one group of controls and the cold-acclimated rats were killed after 14 weeks of training. The trained rats 4 h after their last period of exercise and the cold acclimated rats after 24 h at room temperature. One group of exercised rats and one control group were investigated 6 months after the cessation of physical training.

**Collection of urine.** The determinations of the urinary excretion of CA were made during the 12th and 13th week of the training period. The urine was collected in metabolic cages, where the animals were kept individually over a period of 22 h. The sampling of resting CA excretion was preceded by 24 h of rest in the case of the trained rats and by 3 days in room temperature (24°C) in the case of the cold acclimated rats. The same periods of rest and exposure to room temperature respectively preceded the periods of cold stress which was performed at 4°C. Also when sampling was performed after exercise the animals were first allowed to rest for 2 days or were accustomed to room temperature for 3 days before they were submitted to 2 h of swimming before urine collection at room temperature (cf Östman 1972). After the swimming periods the rats were carefully blotted before the urine collection began. Urine samplings were made simultaneously on rats from the different groups. Five ml of 1 M HCl was added as preservative to the collecting bottles. The funnels of the metabolism cages were rinsed with 10 ml of 1 M HCl after removal of the rats. The washing fluid was added to the urine sample.

### CA extraction and determination

The rats were killed by exsanguination under light ether anaesthesia. The hearts, spleens, submandibular glands and adrenal glands were removed, cleaned and weighed. With the exception of the adrenals all organs were homogenized with an Ultra-Turrax apparatus in 15 ml ice-cold 0.4 M perchloric acid containing 0.1 mM ascorbic acid. The rest of the procedure has previously been described in detail (Söderberg 1968). The adrenal glands were ground with chemically pure quartz sand in 2.5 ml 0.4 M perchloric acid.



III. Body weight and length, heart and adrenal weights and cardiac norepinephrine content 6 months after cessation of regular exercise (Mean  $\pm$  S.E.).

	Initial b.w. (g)	Final b.w. (g)	Final body length (cm)	Heart weight (g)	Heart ratio (g/100 g b.w.)	Heart total NA ( $\mu$ g)	Heart NA concentra- tion ( $\mu$ g/g)	Adrenal weight (mg)
cool rats 12)	182 $\pm$ 2	367 $\pm$ 8	26.9 $\pm$ 0.3	1.56 $\pm$ 0.03	0.273 $\pm$ 0.003	1.17 $\pm$ 0.12	0.76 $\pm$ 0.08	54 $\pm$ 2
trained rats 12)	179 $\pm$ 2	343 $\pm$ 13	27.1 $\pm$ 0.1	1.64 $\pm$ 0.03	0.299 $\pm$ 0.003	1.47 $\pm$ 0.11	0.90 $\pm$ 0.07	54 $\pm$ 1
of significance in Student's <i>t</i> -test								
control	n.s.	n.s.	n.s.	n.s.	p 0.001	n.s.	n.s.	n.s.
trained rats								

acclimated rats, the cold-acclimated animals in their turn also excreted significantly NA than the controls did. A conspicuous difference between trained and cold-acclimated rats was revealed when paired observations from the same individual animals were spaced, it was found that the net decrease in the amount of NA excreted after exercise after subtracting the amount of NA excreted during a rest period) was of the same magnitude in cold-acclimated rats and in controls, but significantly smaller in the trained rats (0.60 $\pm$ 0.07  $\mu$ g NA/22 h, *n* = 9; cold-acclimated, 0.64 $\pm$ 0.06  $\mu$ g NA/22 h, *n* = 8; and 0.35 $\pm$ 0.03  $\mu$ g NA/22 h, *n* = 11; *p* = 0.05).

During cold stress the amounts of both NA and A excreted were markedly increased in all experimental groups as compared with the resting state. The amounts of A excreted

as IV The influence of regular physical training and of cold-acclimation on urinary excretion of catecholamines after exercise and during cold stress (Mean  $\pm$  S.E.).

	During rest		After exercise		During cold stress	
	NA- excretion ( $\mu$ g/22 h)	A- excretion ( $\mu$ g/22 h)	NA- excretion ( $\mu$ g/22 h)	A- excretion ( $\mu$ g/22 h)	NA excretion ( $\mu$ g/22 h)	A excretion ( $\mu$ g/22 h)
normal rats	0.93 $\pm$ 0.03 15	0.14 $\pm$ 0.01 15	1.37 $\pm$ 0.04 <sup>d</sup> -12	0.19 $\pm$ 0.02 <sup>d</sup> 11	2.89 $\pm$ 0.15 <sup>d</sup> -12	0.20 $\pm$ 0.01 <sup>d</sup> -12
trained rats	0.64 $\pm$ 0.03 12	0.12 $\pm$ 0.01 12	0.68 $\pm$ 0.07 <sup>d</sup> 18	0.14 $\pm$ 0.02 18	1.32 $\pm$ 0.08 <sup>d</sup> -12	0.21 $\pm$ 0.02 <sup>d</sup> -12
cold-acclimated rats	0.66 $\pm$ 0.06 11	0.07 $\pm$ 0.01 11	1.17 $\pm$ 0.05 <sup>d</sup> 11	0.16 $\pm$ 0.01 <sup>d</sup> 11	2.41 $\pm$ 0.17 <sup>d</sup> -11	0.21 $\pm$ 0.03 <sup>d</sup> 11
test of significance in Student's <i>t</i> -test						
control	n.s.	n.s.	p 0.001	n.s.	p < 0.01	n.s.
trained	p 0.001	p 0.001	p 0.02	n.s.	p 0.05	n.s.
cold accl.	n.s.	p 0.01	p 0.01	n.s.	n.s.	n.s.

number of observations.

different from resting value *p* 0.05.

different from resting value *p* 0.01.

different from resting value *p* 0.001.

n.s. not significant.

TABLE II Effect of physical training and cold-acclimation on catecholamines in different organs ( $\pm$ S.E.).

	Heart total NA ( $\mu$ g)	Heart NA concentration ( $\mu$ g/g)	Salivary glands total NA ( $\mu$ g)	Salivary glands NA concentration ( $\mu$ g/g)	Spleen total NA ( $\mu$ g)	Adrenals total A ( $\mu$ g)	Adrenals total NA ( $\mu$ g)	
Control rats (n=12)	0.87 $\pm$ 0.03	0.71 $\pm$ 0.04	0.68 $\pm$ 0.03	1.02 $\pm$ 0.04	0.66 $\pm$ 0.03	45.1 $\pm$ 1.3	7.4 $\pm$ 0.4	11
Trained rats (n=12)	1.11 $\pm$ 0.06	0.84 $\pm$ 0.05	0.75 $\pm$ 0.03	1.12 $\pm$ 0.05	0.83 $\pm$ 0.03	51.0 $\pm$ 1.5	9.8 $\pm$ 0.5	11
Cold acclimated rats (n=12)	1.01 $\pm$ 0.03	0.77 $\pm$ 0.02	0.81 $\pm$ 0.04	1.13 $\pm$ 0.07	0.57 $\pm$ 0.03	49.6 $\pm$ 1.8	9.8 $\pm$ 0.5	11
Level of significance in Student's <i>t</i> test								
Controls v trained	p<0.01	p<0.05	n.s.	n.s.	p<0.01	p<0.01	p<0.01	p<0.01
Controls cold accl.	p<0.05	n.s.	p<0.05	n.s.	p<0.05	n.s.	p<0.05	p<0.05
Trained cold accl.	n.s.	n.s.	n.s.	n.s.	p<0.001	n.s.	n.s.	n.s.

n.s. = not significant

*Catecholamine content of the organs (see Table II and III)*

The results confirm our earlier observations (Östman *et al* 1972) that training by itself causes a significant increase in both the total amount and the concentration of NA in heart and in the amounts of A and NA in the adrenal glands. However in the adrenal glands the increase in NA-content is relatively greater than that of A as shown by the fact that the relative proportion of NA is significantly increased. A significantly higher content was also found in the spleens of the trained rats. Cold-acclimation led to a significant increase in cardiac NA-content, but in contrast to trained rats, the increase in cardiac NA-concentration was not significant. Increased NA-content but not NA-concentration was also found in the submandibular glands of the cold-acclimated animals, but the NA-content of the spleens, on the other hand was significantly decreased. In the adrenals of cold-acclimated rats only the NA-content was significantly increased and as with the trained rats the proportion of the total CA in the adrenal glands made up by NA was significantly increased. Six months after cessation of physical training no significant difference existed between the cardiac NA-contents of the ex-trained rats and those of the controls (Table

*Urinary CA-excretion (see Table IV and V)*

During resting conditions both the trained and the cold-acclimated rats excreted significantly less NA than the controls did. Furthermore, the cold-acclimated rats also excreted significantly less A than did both the controls and the trained rats.

In the urine collected after exercise all groups showed a significantly increased NA-excretion as compared with the resting state, but only the controls and the cold-acclimated rats showed a significant increase in A-excretion. The total amount of NA excreted after exercise by the trained rats, however was significantly lower than that of both control

### catecholamine content of tissues

previous finding of increased cardiac NA-content as well as NA-concentration in electrically exercised rats (Östman *et al.* 1972) is confirmed in this study and we also find an increased content of NA in the spleen, another organ involved in adjustments to conditions of regulatory stress. The increase in NA is probably related to more effective mechanisms of release and storage of the transmitter induced by the daily exercise as discussed by Östman *et al.* (1972). Thus, an increased impulse activity in the sympatho-adrenal system induces increased activity in ganglia and organs of tyrosine hydroxylase (cf. Thoenen *et al.* 1973) as well as of dopamine  $\beta$ -hydroxylase (Mollnoff *et al.* 1970) which is a component of the storage vesicle (Smith 1972) and therefore also may reflect an increased storage capacity for NA.

De Schryver and co-workers (cf. De Schryver and Mertens-Strythagen 1972) have reported increased cardiac NA-content in "trained" rats and suggested that this could in part explain the bradycardia of the athletic heart. However, these authors use a very low training intensity (running for 90 min at a speed of 8.3 or 12.5 m/min) and they exercise their rats only three times a week. Such training parameters are not sufficient to induce cardiac hypertrophy (Petow and Siebert 1925, Leon and Bloor 1968). Accordingly De Schryver and associates never see any sign of cardiac hypertrophy in their "trained" rats. We find their data on cardiac NA-content puzzling but since the athletic heart by definition is an enlarged heart and since De Schryver and co-workers never measured the heart rate in their rats, we reject their conclusion that their finding of reduced transmitter levels should in part explain the bradycardia of the athletic heart (see also Östman and Sjöstrand 1971).

The cardiac hypertrophy of the cold-acclimated rats is presumably related to the increased cardiac output occurring after cold-exposure (Jansky and Hart 1968, Popovic *et al.* 1969), and the increased cardiac NA due to an increased activity in the sympathetic nerves as discussed above. Thus, cold exposure increases cardiac NA-turnover just as does exercise (Jordan *et al.* 1966).

The decreased NA-content in the spleen of cold-acclimated rats might be due to a decreased sympathetic activity in the organ caused by the increased peripheral vasoconstriction combined with high circulating levels of CA. Thus, the data of Jansky and Hart (1968) indicate that the spleen, in contrast to most other vascular beds, is not actively involved in the regulatory adjustments following acute or chronic exposure to cold.

The increased adrenal CA levels and the increase in relative proportion of NA found in trained and cold-acclimated rats are in agreement with earlier findings (Östman *et al.* 1972, Kvotnansky *et al.* 1971). Also the increase in adrenal CA-content is likely to be mediated through induction of enzymes (cf. Mollnoff *et al.* 1970, Kvotnansky *et al.* 1971).

### renal excretion of catecholamines

*Trained rats.* The previous observation of a smaller increase in urinary NA excretion after exercise in trained rats than in controls is confirmed in this study. This time we also find a lower NA-excretion in trained rats during rest (i.e. at least 48 h after the last exercise). Our results suggest that chronically exercised animals require a lower degree of sympathetic tone in order to maintain homeostasis in rest as well as during exercise. This fits with the

TABLE V Urinary excretion of catecholamines 6 months after cessation of regular exercise (New 1)

	During rest		After exercise		During cold stress	
	NA excretion ( $\mu\text{g}/22 \text{ h}$ )	A excretion ( $\mu\text{g}/22 \text{ h}$ )	NA excretion ( $\mu\text{g}/22 \text{ h}$ )	A excretion ( $\mu\text{g}/22 \text{ h}$ )	NA excretion ( $\mu\text{g}/22 \text{ h}$ )	A excretion ( $\mu\text{g}/22 \text{ h}$ )
Old control rats	$1.12 \pm 0.05$ $n=14$	$0.14 \pm 0.01$ $n=14$	$1.73 \pm 0.09^d$ $n=14$	$0.44 \pm 0.03^b$ $n=14$	$3.74 \pm 0.12^d$ $n=14$	$0.21 \pm 0.01$ $n=14$
Ex trained rats	$1.03 \pm 0.06$ $n=14$	$0.11 \pm 0.01$ $n=14$	$1.46 \pm 0.07^d$ $n=14$	$0.22 \pm 0.02^d$ $n=14$	$3.39 \pm 0.16^d$ $n=14$	$0.18 \pm 0.01$ $n=14$

Level of significance in Student's *t*-test

Old control rats - ex trained	n.s.	n.s.	$p < 0.05$	n.s.	n.s.	n.s.
-------------------------------	------	------	------------	------	------	------

$n$  = number of observations.

$^b$  = different from resting value  $p < 0.05$ .

$^d$  = different from resting value  $p < 0.001$ .

n.s. = not significant

were of the same order of magnitude in all three groups the amounts of NA excreted both the cold-acclimated rats and the trained rats were, however significantly lower than the amounts excreted by the controls.

Six months after cessation of swimming training the only significant difference between control and ex trained rats was that the ex trained rats excreted less NA stress than did the controls (Table V). This was also found when the net increase in the amount of NA excreted by individual rats after exercise was determined in paired observations: controls  $0.75 \pm 0.10 \mu\text{g NA}/22 \text{ h}$ ,  $n=10$  ex-trained  $0.41 \pm 0.07 \mu\text{g NA}/22 \text{ h}$ ,  $n=11$   $p < 0.05$ .

### Discussion

#### Organ weights

The increase in heart and adrenal weights agree with earlier findings in trained (Hart) and cold acclimated (Emery *et al.* 1940, Heroux and Hart 1954) rats. However in respects the present data diverge from earlier findings. Firstly we do not see the inhibition of growth of cold-acclimated animals reported by e.g. Schwabe *et al.* (1938), Heroux and Hart (1954). This might be due to the fact that in our experiment the effect of the cold stress is diminished by keeping the rats in groups and in furnished cages.

Secondly our data indicate that a certain degree of cardiac hypertrophy persists at least 6 months after cessation of training. Other investigators e.g. Secher (1923), Hort (1951), Leon and Bloor (1968) and Ljungquist and Ungo (1972), have reported a rapid return of heart weight to control values when chronic exercise is discontinued. The discrepancy is probably explained by the fact that we have trained male rats rather intensely during puberty while other authors have used old rats (Hort 1951, Ljungquist and Ungo 1972) or exercised the rats less severely (Leon and Bloor 1968).

The increase in weight of the salivary gland of the cold-acclimated rats is probably related to an increased food intake (*cf.* Schwabe *et al.* 1938) and might be caused by an increased activity in the sympathetic nerves to the gland (*cf.* Himms-Hagen 1972).

that chronically exercised rats have a lower demand for NA both in the warm and in cold, and that they have greater tissue reserves of CA together with a greater capacity for synthesis of CA, we suggest that increased levels of physical activity in mammals may lead to a better fitness to withstand cold and, possibly, other stressful conditions.

This work was supported by grants from the Swedish Medical Research Council (Nos. 873-04X-3474) and Lars Hiertas Minne. We are deeply grateful to Mrs. Kerstin Westling for her excellent technical assistance and to Mrs. A. Rosen for patient preparation of the manuscript.

## References

1. C. C., A reliable method for spectrophotofluorimetric assay of catecholamines. *Int. J. Neuropharmacol.* 1964, 3, 643-649.
2. J. F., The catecholamine response of cold-acclimated white rats to infused noradrenaline. *Canad. J. Physiol. Pharmacol.* 1980, 58, 107-114.
3. J. F., C. and J. M., The catecholamine response of cold-acclimated white rats to infused noradrenaline. *Canad. J. Physiol. Pharmacol.* 1972, 50, 345-354.
4. F. E., L. M., E. and E. L., The effects of prolonged exposure to low temperatures on body growth and on the weights of organs in the albino rat. *Growth* 1940, 4, 17-32.
5. J. F., R. S., S. and S., Increased synthesis of noradrenaline and dopamine in the intact rat during exercise and exposure to cold. *J. Pharmacol. exp. Ther.* 1966, 151, 447-457.
6. J. F., M. H., E., L. and R. T., Changed chromotropic sensitivity to sympathomimetic amines in isolated aorta from rats following cold acclimation. *Experientia (Basel)* 1974, 30, 1041-1043.
7. J. F., On the influence of exercise on the growth of organs in the albino rat. *Amer. J. Zool.* 1915, 9, 647-665.
8. J. F., Comparison between seasonal and thermal acclimation in white rats. *Canad. J. Biochem. Physiol.* 1961, 39, 1829-1836.
9. J. F., O. and J. S., Comparison of four indices of adrenal activity in rats acclimated to 30°C. *Amer. J. Physiol.* 1954, 178, 445-448.
10. J. F., Effects of catecholamines on metabolism. VIII. Salivary glands. In: *Handbook of Experimental Pharmacology XXXIII Catecholamines*. Eds. H. Blaschko and E. Blaschko, pp. 423-427. Springer-Verlag, Berlin, Heidelberg, New York, 1972.
11. J. F., Role of the adrenal medulla in adaptation to cold. In: *Handbook of Physiology Section 1*, Vol. 6, 1973.
12. J. F., M. H., J. and I. M., Unchanged sensitivity to noradrenaline in isolated aorta from cold-acclimated rats. *Canad. J. Physiol. Pharmacol.* 1970, 48, 657-660.
13. J. F., Morphologische und physiologische Untersuchungen an Ratten. Abhandlung 1. *Lehrbuch der Tierphysiologie*. 1951, 220, 197-237.
14. J. F., A. C. L. and L. D., Role of adrenaline and noradrenaline in chemical regulation of heart function. *Amer. J. Physiol.* 1957, 190, 243-246.
15. J. F., A. C. L., C. W., K. M. and K. W., Circulatory and metabolic effects of noradrenaline in cold-acclimated rats. *Fed. Proc.* 1966, 25, 1205-1212.
16. J. F., R. BARTUNOV and E. ZIMMERMAN, Acclimation of the white rat to cold. Noradrenaline thermogenesis. *Pharmacol. Biochem. Behav.* 1967, 16, 366-372.
17. J. F. and J. S. H., Cardiac output and organ blood flow in warm- and cold-acclimated rats exposed to cold. *Canad. J. Physiol. Pharmacol.* 1968, 46, 653-659.
18. J. F., R. G., P. G. and V. K., Catecholamine-synthesizing enzymes in the rat adrenal gland during exposure to cold. *Amer. J. Physiol.* 1971, 220, 928-931.
19. J. F., Effect of adrenaline, noradrenaline and chlorpromazine on blood pressure of normal and cold-acclimated rats. *Proc. Soc. exp. Biol. (N.Y.)* 1960, 105, 108-111.
20. J. F. and M. P., Importance of noradrenaline in cold adaptation. *Amer. J. Physiol.* 1964, 207, 833-836.
21. J. F., J. VALLBOM and C. VALLBOM, Beta-receptor sensitization by repeated injections of isoprenaline and by cold adaptation. *Amer. J. Physiol.* 1972, 222, 1043-1046.

decreased NA turnover found in the heart (Östman *et al.* 1972) and the spleen (Östr. Nybäck 1975) of trained rats and also with the increased vascular sensitivity to NA in these rats (Östman 1975).

The reduced NA excretion during cold stress of the trained rats might in part be due to a lower need for the transmitter in the cardiovascular adjustments that occur during exposure. However it is also quite possible that chronic exercise increases the sensitivity of the tissues to the metabolic action of NA. This could be due to the daily exposure of the rats to higher than normal concentrations of CA during the exercise periods. Thus, the treatment of rats with NA sensitizes their tissues to the metabolic actions of NA (LeBlanc Poullot 1964).

Six months after cessation of exercise the trained rats still excreted less NA than controls. Possibly this reflects the persistence of reduced sympathetic tone to the hypertrophied heart and, perhaps, also a better circulatory adjustment, rather than a persistent increase in vascular and/or metabolic sensitivity to the transmitter. Thus, the increased tissue sensitivity to the metabolic actions of NA of cold-acclimated rats declines rather rapidly when the rats are retransferred to warm environment (Jansky *et al.* 1967).

**Cold-acclimated rats** The depressed levels of urinary CA in these animals when kept at room temperature, as well as their decreased NA excretion during cold stress, are probably to a great extent related to the increased sensitivity to the calorogenic effects of NA which occurs in cold-acclimation (*cf.* Hinms-Hagen 1975). However the cold-acclimated rats had the same net increment in NA excretion after exercise as the controls had. It appears that cold-acclimated rats do not develop any "cross-tolerance" to the cardiovascular (and metabolic) stress of exercise, although chronically exercised rats do seem to develop a degree of "cross-tolerance" to the metabolic (and circulatory) stress of cold exposure. Reasons for this difference are not known. Both groups develop cardiac hypertrophy. Conflicting results have been reported concerning cardiovascular sensitivity to NA in acclimated rats. Increased sensitivity or increased maximum response to CA of heart vessels have been claimed by some investigators (LeBlanc 1960, Heroux 1961, LeBlanc 1972, Harri *et al.* 1974) while other authors have denied such effects (Haleh *et al.* 1970, Hinms-Hagen and Mazurkiewicz Kwilecki 1970). Our results are more consistent with the latter view but other explanations can not be excluded.

### Conclusions

The adaptive changes following physical training include a greater economy of the sympathetic transmitter which may well be to a large extent due to increased sensitivity of the tissues to the vascular and metabolic actions of NA. The consequences of this are obvious: the animal develops a better tolerance to conditions that require an increased transmitter secretion in order to maintain homeostasis. Exposure to severe cold is, for a small mammal, a critical situation and it is the capacity for non-shivering thermogenesis induced by the calorogenic actions of CA that determines the survival of such an animal. In natural conditions the activity period is increased during the autumn and winter for a nocturnal species such as the rat. Since our results in this and previous studies

# Effects of Drugs and Nerve Stimulation on the Spleen and Arteries of Two Species of Dogfish, *Scyllorhinus canicula* and *Squalus acanthias*

By

STEFAN NILSSON, SUSANNE HOLMGREN and DAVID J. GROVE

Received 26 March 1975

## Abstract

NILSSON, S., HOLMGREN, S. and GROVE, D. J. Effects of drugs and nerve stimulation on the spleen and arteries of two species of dogfish, *SCYLLORHINUS CANICULA* and *SQUALUS ACANTHIAS*. Acta physiol. scand. 1975. 95. 219-230.

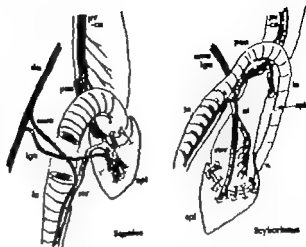
Effects of drugs and nerve stimulation on the spleen of 2 species of dogfish have been examined by means of perfused spleens and isolated spleen and artery strips. Adrenaline, noradrenaline and typhine, acting via alpha adrenoreceptors, contract the perfused spleen of both species, thereby releasing erythrocytes, and contract the isolated spleen and artery strips. Phentolamine competitively antagonizes the excitatory effects of adrenergic agonists. The responses of the spleen to acetylcholine are very slow and rapid desensitization makes evaluation of the mode of action of this drug difficult. In the 2 strips acetylcholine produces dose-dependent contraction. Fluorescent histochemistry reveals all splenic adrenergic innervation of the arteries, and few adrenergic terminals in the spleen. Stimulation of these nerves produces normally splenic constriction in *Squalus*, which can be blocked by phentolamine but not by typhine. The nervous control of the *Scyllorhinus* spleen seems to be poor or lacking. It is concluded that the dogfish spleen, and possibly also the arteries, are to a large extent controlled by circulating cholinergic and (in *Squalus*) also by sympathetic adrenergic fibres.

Keywords: Dogfish, spleen innervation, autonomic nervous system, adrenergic drugs, cholinergic drugs

There are few reports concerning the effects of drugs on and autonomic control of the spleen of fish. Acetylcholine and alpha adrenoreceptor stimulating agents contract isolated spleen strips from the tench (*Tinca tinca*) (Vairé 1933) and the cod (*Gadus morhua*) (Holmgren and Nilsson 1975). Bonnet (1929) concluded from her experiments with the tench that the spleen of fish releases erythrocytes in response to asphyxia, and Nilsson and Grove (1974) showed that in the perfused spleen of cod, splenic constriction and release of erythrocytes can be induced both by nerve stimulation and by injection of acetylcholine or alpha adrenoreceptor agonists. The nerve supply to the cod spleen consists of both adrenergic and cholinergic outganglionic sympathetic constrictory fibres (Nilsson and Grove 1974). Opdyke and Opdyke (1971) concluded by examining the haematocrit of the splenic effluent in the spiny

- LEDUC, J. Catecholamine production and release in exposure and acclimation to cold. *Acta physiol.* 1961 53 Suppl. 183.
- LEON, A. S. and C. M. BLOOM, Effects of exercise and its cessation on the heart and its blood. *appl. Physiol.* 1968. 24 485-490.
- LIUNGGUIST, A. and G. UNGE, The finer intramyocardial vasculature in various forms of myocardial hypertrophy. *Acta path. microbiol. scand.* 1972. Section A 80 329-340.
- MOLDOFF, P. B., S. BRIMMON, R. WEINSHILBOUM and J. AXELROD, Neurally mediated histamine- $\beta$ -hydroxylase activity. *Proc Nat Acad. Sci.* 1970. 66. 453-458.
- ÖSTMAN, I., Changed vascular sensitivity to noradrenaline in rats trained by physical exercise. *Jt (Lond.)* 1975. In press.
- ÖSTMAN, I. and H. NYRÅCK, Adaptive changes in central and peripheral noradrenergic systems following chronic exercise. *Neuroscience* 1975. In press.
- ÖSTMAN, I. and N. O. SÖSTRAND, Effect of prolonged physical training on the catecholamine in the heart and the adrenals of the rat. *Acta physiol. scand.* 1971 82. 20. 708.
- ÖSTMAN, I., N. O. SÖSTRAND and G. SWEDIN, Cardiac noradrenaline turnover and urinary catechol excretion in trained and untrained rats during rest and exercise. *Acta physiol. scand.* 1972 83. 3.
- PITROW, H. and W. SIEMERT, Studien über Arbeitshypertrophie des Muskels. *Z. Klin. Med.* 185 427-433.
- POPOVIC, V. K., KENT N. MOJAVIC, B. MOJAVIC and J. S. HART, Effect of exercise and cold on output in warm- and cold-acclimated rats. *Fed. Proc.* 1969 28. 1138-1141.
- SCHWABE, E. L., F. E. EMERY and F. R. GRIFFITH Jr, The effect of prolonged exposure to low temperature on the basal metabolism of the rat. *J. Amer.* 1938. 15 199-210.
- SECHER, K., Experimentelle Untersuchungen über die Grösse des Herzens nach einem Anstoss an Nerven. *Z. exp. Med.* 1923. 32. 790-795.
- SMITH, A. D., Subcellular localization of noradrenaline in sympathetic neurons. *Pharmacol. Rev.* 24 435-457.
- SWEDIN, G. Studies on neurotransmission mechanisms in the rat and guinea-pig vas deferens. *Acta physiol. scand.* 1971 83 Suppl. 369.
- THOMPSON, H., U. OTTEN and H. ORICI, Trans-synaptic regulation of tyrosine hydroxylase. In *Folia Catecholaminica Research*. Eds. E. Usdin and S. Snyder. pp. 179-185. Pergamon Press, Oxford 1974.





Schematic diagram of the  
ry of the blood vessels  
the spleen in *Squalus*  
and *Scyliorhinus* com-  
pared with anterior mes-  
enteric ca, coeliac artery,  
celiac axis, splenic artery,  
splenic vein; lpa, portal  
vein, splenic vein, splenic  
artery.

*Squalus*

*Scyliorhinus*

the following drugs were used: acetylcholine chloride, L-adrenaline bitartrate, atropine sulphate, car-  
bachol chloride, 5-hydroxytryptamine creatinine sulphate, DL-isoprenaline hydrochloride, metacetyl-  
choline chloride, nicotine sulphate, L-noradrenaline bitartrate, phenolamine methanesulphate, pro-  
pranolol hydrochloride, propranolol hydrochloride, salbutamol chloride. The amounts or con-  
centrations of these drugs are expressed in moles or mol/l (M) respectively.

#### Microhistology

of the hepatic artery the coeliac artery the spleen and sympathetic ganglia from *Squalus* were  
fixed in liquid propane cooled in liquid nitrogen and freeze-dried according to the method described  
by Wick and Olszewski (1965). One animal was injected with noradrenaline (3 mg/kg) 1 h prior to killing and  
after the organs. After treatment with formaldehyde at 80°C for 1 or 3 h, the preparations were  
fixed in paraffin wax. Controls were embedded without previous formaldehyde treatment. Sections  
were mounted in Eutectan (Merck) and viewed in a Leitz Orthomat microscope with top illumination  
by a barrier filter at 460 nm. Photographs were taken by a Leitz Orthomat automatic camera on Kodak  
Ektachrome film.

## Results

### Effects of isolated strip preparations

energetic drugs. Adrenaline, noradrenaline and phenylephrine contracted the spleen strips  
in both *Scyliorhinus* and *Squalus* as well as the artery strips from *Squalus* (Table I, Fig. 6).  
Artery strips were also contracted by adrenaline and noradrenaline (Table I),  
very few  $pD_{50}$  values have been calculated since in most preparations the first low dose of  
agent induced rhythmic activity which could not be abolished, and no dose-response  
curves could be obtained on these preparations.

Adrenaline ( $10^{-7}$ – $10^{-5}$  M) had no detectable effect on the spleen strips from either  
species. Concentrations between  $10^{-7}$  M and  $10^{-5}$  M slightly relaxed artery strips from  
both, while higher doses caused a small contraction.

The alpha adrenoreceptor antagonist phentolamine produced a parallel shift of the dose-  
response curves for noradrenaline in the *Scyliorhinus* spleen strips and the *Squalus* spleen and  
artery strips. The  $pA_{50}$  values are summarized in Table II.

dogfish (*Squalus acanthias*) that this species has not developed the ability to release lymphocytes from the spleen in response to sympathetic nerve stimulation or adrenaline.

The present work examines the mode of autonomic innervation and the effects of adrenergic and cholinergic drugs on the spleen of dogfish by experiments with isolated splenic artery strips and perfused spleens.

## Materials and Methods

Dogfish, *Scyliorhinus canicula* (650–750 g, length 60–65 cm) and spiny dogfish, *Squalus acanthias* (1400 g, length 50–75 cm) of either sex were used in the experiments. The animals were kept in well-aerated sea water at 10–15°C for less than 4 weeks before the experiments.

### Experiments with isolated strip preparations

Spleen strips, approx. 25 mm long and 2–3 mm wide including parts of the capsule, or longitudinal strips of the meso-gastric or coeliac artery (Fig. 1) were suspended in organ baths and attached to GRASS isometric transducers connected to a GRASS Polygraph model 7. The organ baths contained a saline of the following composition (*Scyliorhinus*): NaCl 15.05, NaHCO<sub>3</sub> 2.20, Na<sub>2</sub>HPO<sub>4</sub> 0.23, KH<sub>2</sub>PO<sub>4</sub> 0.05, KCl 0.4, CaCl<sub>2</sub> 6.11, D-glucose 1.0 and urea 4.0 g/litre or (*Squalus*): NaCl 13.5, KCl 0.4, CaCl<sub>2</sub> 2.1, D-glucose 0.70, MgSO<sub>4</sub> 7.11, D-glucose 0.60, NaHCO<sub>3</sub> 1.00, Na<sub>2</sub>HPO<sub>4</sub> 2.11, D-glucose 1.0 and urea 4.0 (calculated from *Squalus* plasma levels from Holmes and Donaldson 1969). The saline was kept at room temperature within the range 10–15°C and bubbled with a mixture of O<sub>2</sub> and CO<sub>2</sub> (*Scyliorhinus* 95.5% O<sub>2</sub>, *Squalus* 97.3% O<sub>2</sub>, giving a pH of ca. 7.5 in both cases). The tension of the spleen strips was adjusted to 50 mg, and the arteries were adjusted to their in vivo length. The preparations were left to reach a baseline for 1 h (*Scyliorhinus*) or up to 18 h (*Squalus*) before any addition of drugs.

Cumulative dose-response curves were obtained and pD<sub>50</sub>-values ( $pD_{50} = -\log ED_{50}$ , as Rossum 1963 determined for the various agonists. Experiments, where subsequent cumulative dose-response curves to one agonist were produced, showed that the pD<sub>50</sub>-values did not differ detectably from one curve to another but that the maximal contraction force obtained was very irregular. Consequently it has not been possible to evaluate the intrinsic activity (alpha-value, van Rossum 1963) for the different agonists and their effects on the preparations will be presented only as their pD<sub>50</sub>-values.

In the experiment with the alpha-adrenoceptor antagonist phentolamine, this was added to the bath 10 min before obtaining the second (or third) dose-response curve. The pA values are determined according to Benfey and Grillo (1963) as described by Holmgren and Nilsson (1975).

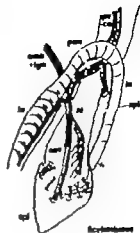
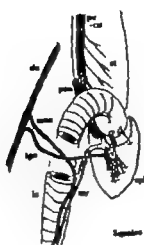
### Experiments with perfused spleen

The animals were injected with 1000–2000 I.U. of heparin in the caudal vessels a few min before the experiment by a sharp blow on the head and subsequent pinning. The spleen was exposed and the splenic artery and vein (*Scyliorhinus*) or meso-gastric artery and testal vein (*Squalus*) (Fig. 1) cannulated for the inflow and outflow respectively of perfusion fluid. All vessels entering and leaving the spleen as well as the cannulae were carefully ligated. The spleen was separated from the stomach, removed from the abdominal cavity and allowed to rest on tissues moistened with saline. The *Scyliorhinus* spleen was ligated at the constriction between the two parts of the spleen (Fig. 1, left arrow) to allow perfusion of the posterior (main) part.

The perfusion fluid was the saline described above, and in the case of *Scyliorhinus*, 15 g polyvinylpyrrolidone per litre was added to avoid edema (Davies and Rankin 1973) but this was found unnecessary in *Squalus*. Perfusion was carried out at a constant pressure (variation during each experiment less than 0.5 cm H<sub>2</sub>O) within the range 25–30 cm H<sub>2</sub>O with the apparatus of Davies and Rankin (1973) as modified by Nilsson and Grove (1974). The outflow pressure was close to zero cm H<sub>2</sub>O. Perfusion flow was determined as drop rate either on the inflow and outflow side by GRASS FT1 photoelectric transducers connected to a GRASS 7P4 tachograph, or on the outflow side only by a drop-counter similar to that described by Davies and Rankin (1973). Drugs were added either as single doses by a syringe and a catheter into the post-splenic stream or dissolved in the perfusion fluid. Responses were determined as the flow decrease in per cent from the initial flow rate measured on the outflow side.

Stimulation of the splenic nerves were made by two silver electrodes over which the whole spleen was placed. Trains of pulses lasting up to 120 s with 10–50 Hz, 0.5–1.0 ms duration and 3–18 V were applied.

Simplified diagram of the  
y of the blood vessels  
the spleen in *Squalus*  
is and *Scyliorhinus* con-  
sponds also, anterior meso-  
artery ca, coeliac artery  
and aorta, sa, spiral aorta,  
sc, cranial vein, lga,  
spleen artery paa, pan-  
or portal vein, arv spleen  
and vein, spl, spleen, st,  
st.



following drugs were used. acetylcholine, chloride, L-adrenaline bitartrate, atropine sulphate, car-  
ne chloride, 5-hydroxytryptamine creatinine sulphate, DL-noradrenaline hydrochloride, mephenter-  
mine hydrochloride, mephentermine sulphate, L-noradrenaline bitartrate, phenylephrine hydrochloride,  
propionolol hydrochloride, salbutamol chloride. The amounts or con-  
centrations of these drugs are expressed in moles or mol/l (M) respectively

# *Scyliorhinus*

all of the meso-gastric artery the coeliac artery the spleen and sympathetic ganglia from *Squalus* were  
excised in liquid propane cooled in liquid nitrogen and freeze-dried according to the method described  
by J. L. and O. (1965). One animal was injected with noradrenaline (5 mg/kg) 1 h prior to killing and  
fixing the organs. After treatment with formaldehyde vapour at 80°C for 1 or 3 h, the preparations were  
fixed in paraffin wax. Controls were embedded without previous formaldehyde treatment. Sections  
5  $\mu$ m were mounted in Eutectic (Merk) and viewed in a Leitz Ortholux microscope with top illumination  
at lower light at 460 nm. Photographs were taken by a Leitz Orthomat autographic camera on Kodak  
film.

## Results

### *Scyliorhinus* and *Squalus* isolated strip preparations

Adrenaline, noradrenaline and phenylephrine contracted the spleen strips  
from both *Scyliorhinus* and *Squalus* as well as the artery strips from *Squalus* (Table I, Fig. 6).  
Spleen artery strips were also contracted by adrenaline and noradrenaline (Table I).  
Very few  $pD_{50}$ -values have been calculated since in most preparations the first low dose of  
agonist induced rhythmic activity which could not be abolished, and no dose-response  
curves could be obtained on these preparations.  
Noradrenaline ( $10^{-6}$ – $10^{-8}$  M) had no detectable effect on the spleen strips from either  
species. Concentrations between  $10^{-6}$  M and  $10^{-8}$  M slightly relaxed artery strips from  
*Squalus*, while higher doses caused small contraction.  
The  $\alpha$ -adrenoceptor antagonist phentolamine produced a parallel shift of the dose-  
response curve for noradrenaline in the *Scyliorhinus* spleen strips and the *Squalus* spleen and  
artery strips. The  $pA$ -values are summarized in Table II.

dogfish (*Squalus acanthias*), that this species has not developed the ability to release lymphocytes from the spleen in response to sympathetic nerve stimulation or adrenaline.

The present work examines the mode of autonomic innervation and the effects of adrenergic and cholinergic drugs on the spleen of dogfish by experiments with isolated spleen artery strips and perfused spleens.

## Materials and Methods

Dogfish, *Scyliorhinus canicula* (650–750 g, length 60–65 cm) and spiny dogfish, *Squalus acanthias* (1400 g, length 50–75 cm) of either sex were used in the experiments. The animals were kept in a well-aerated sea water at 10–15°C for less than 4 weeks before the experiments.

### Experiment 1: Isolated strip preparations

Spleen strips, approx. 25 mm long and 2–3 mm wide including parts of the capsule, or longitudinal sections of the ileo-gastric or coeliac artery (Fig. 1) were suspended in organ baths and attached to Grass isometric transducers connected to a GRASS Polygraph model 7. The organ baths contained a salt solution following composition (*Scyliorhinus*): NaCl 15.05, NaHCO<sub>3</sub> 2.20, Na<sub>2</sub>HPO<sub>4</sub> 0.23, K<sub>2</sub>HPO<sub>4</sub> 0.05, KCl 0.40, CaCl<sub>2</sub> 6H<sub>2</sub>O 1.10, MgSO<sub>4</sub> 7H<sub>2</sub>O 1.00, glucose 1.0 and urea 24.0 g/litre or (*Squalus*): NaCl 13.5, KCl 0.40, CaCl<sub>2</sub> 2H<sub>2</sub>O 0.70, MgSO<sub>4</sub> 7H<sub>2</sub>O 0.60, NaHCO<sub>3</sub> 1.00, Na<sub>2</sub>HPO<sub>4</sub> 2H<sub>2</sub>O 0.24, glucose 1.0 and urea 24.0 (calculated from *Squalus* plasma levels from Holmes and Donaldson 1969). The solution was kept at a temperature within the range 10–15°C and bubbled with a mixture of O<sub>2</sub> and CO<sub>2</sub> (*Scyliorhinus* 97.3% giving a pH of c.a. 7.5 in both cases). The tonus of the spleen strips was adjusted to 50 mg, and the arteries were adjusted to their *in situ* length. The preparations were left to reach a base-line for 1 h (*Scyliorhinus*) or up to 18 h (*Squalus*) before any addition of drugs.

Cumulative dose-response curves were obtained and pD<sub>50</sub>-values (pD<sub>50</sub> = -log ED<sub>50</sub>) were determined for the various agonists. Experiments, where subsequent cumulative dose-response curves to one agonist were produced, showed that the pD<sub>50</sub>-values did not differ detectably from one curve to another but that the maximal contraction force obtained was very irregular. Consequently it has been possible to evaluate the intrinsic activity (alpha-value, van Rossum 1963) for the different agonists and their effects on the preparations will be presented only as their pD-values.

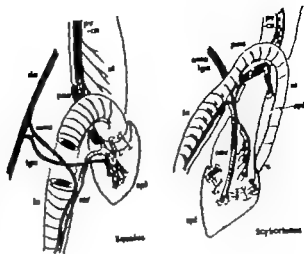
In the experiments with the alpha-adrenoceptor antagonist phentolamine, this was added to the bath 10 min before obtaining the second (or third) dose-response curve. The pA-values were determined according to Bentley and Grillo (1963) as described by Holmgren and Nilsson (1975).

### Experiment 2: In perfused spleen

The animals were injected with 1 000–2 000 I.U. of heparin in the caudal vessels a few min before being anaesthetized by a sharp blow on the head and subsequent pithing. The spleen was exposed and the splenic artery in (*Scyliorhinus*) or ileo-gastric artery and intestinal vein (*Squalus*) (Fig. 1) catheterized for the inflow and outflow respectively of perfusion fluid. All vessels entering and leaving the spleen as well as the coeliac vessels were carefully ligated. The spleen was separated from the stomach, removed from the animal and allowed to rest on tissues moistened with saline. The *Scyliorhinus* spleen was ligated at the connection between the two parts of the spleen (Fig. 1, arrow) to allow perfusion of the posterior (mesal) part.

The perfusion fluid was the saline described above, and in the case of *Scyliorhinus*, 15 g polyvinylpyrrolidone per litre was added to avoid edema (Davies and Rankin 1973) but this was found unnecessary in *Squalus*. Perfusion was carried out at a constant pressure (variation during each experiment less than 5 mm Hg) within the range 25–30 cm H<sub>2</sub>O with the apparatus of Davies and Rankin (1973) as modified by Nilsson and Grove (1974). The outflow pressure was close to zero cm H<sub>2</sub>O. Perfusion flow was determined as drop rate either on the inflow and outflow side by GRASS PT1 photoelectric transducers connected to a GRASS 7P4 tachograph, or on the outflow side only by a drop-counter similar to that described by Davies and Rankin (1973). Drugs were added either as single doses by syringe and a catheter into the perfusion stream or dissolved in the perfusion fluid. Responses were determined as the flow decrease as per cent of the initial flow rate measured on the outflow side.

Stimulation of the splenic nerves was made by two pairs of electrodes over which the whole spleen was placed. Trains of pulses lasting up to 120 s with 10–50 Hz, 0.5–1.0 ms duration were applied.



simplified diagram of the of the blood vessels the spleen in *Squalus* and *Scyliorhinus* (anterior vena, anterior mesenteric ca., coeliac artery, aorta, in. splenic artery, splenic vein, hepatic artery, portal vein, splenic vein; ap, spleen, st.

following drugs were used: acetylcholine, chloride, L-adrenaline bitartrate, atropine sulphate, carbachol, 5-hydroxytryptamine creatinine sulphate, DL-isoprenaline hydrochloride, procainamide hydrochloride, atropine sulphate, L-noradrenaline bitartrate, phentolamine methane sulphate, propranolol hydrochloride, salbutamol chloride. The amounts or concentrations of these drugs are expressed in moles or mol/l (M) respectively

of the lino-gastric artery the coeliac artery the spleen and sympathetic ganglia from *Squalus* were frozen in liquid propane cooled in liquid nitrogen and freeze-dried according to the method described by Lick and Orison (1965). One animal was injected with noradrenaline (3 mg/kg) 1 h prior to killing and leaving the organs. After treatment with formaldehyde vapour at 80°C for 1 or 3 h, the preparations were embedded in paraffin wax. Controls were embedded without previous formaldehyde treatment. Sections (5 µm) were mounted in Eucaline (Merck) and viewed in a Leitz Ortholux microscope with top illumination. A 10x objective lens was used at 460 nm. Photographs were taken by a Leitz Orthomat automatic camera on Kodak film.

## Results

### Isolated strip preparations

**Adrenergic drugs.** Adrenaline, noradrenaline and phenylephrine contracted the spleen strips from both *Scyliorhinus* and *Squalus* as well as the artery strips from *Squalus* (Table 1, Fig. 6). *Scyliorhinus* artery strips were also contracted by adrenaline and noradrenaline (Table 1), but very few  $PD_{50}$ -values have been calculated since in most preparations the first low dose of agonist induced rhythmic activity which could not be abolished, and no dose-response curves could be obtained on these preparations. Isoprenaline ( $10^{-6}$ – $10^{-5}$  M) had no detectable effect on the spleen strips from either species. Concentrations between  $10^{-6}$  M and  $10^{-5}$  M slightly relaxed artery strips from *Squalus*, while higher doses caused a small contraction. The alpha adrenoceptor antagonist phentolamine produced a parallel shift of the dose-response curve for noradrenaline in the *Scyliorhinus* spleen strips and the *Squalus* spleen and artery strips. The  $PA$ -values are summarized in Table 11.

TABLE I Affinity ( $pD_5$ ) values for the different agonists tested on spleen strips and coeliac and/or artery strips from *Scyliorhinus canicula* and *Squalus acanthias*. The values are given  $\pm$  S.E. Numbers within parentheses indicate number of experiments.

	Adrenaline	Noradrenaline	Phenylephrine	Acetylcholine
<i>Scyliorhinus</i>				
Spleen	$6.19 \pm 0.09$ (7)	$5.59 \pm 0.08$ (16)	$5.47 \pm 0.05$ (4)	no response
Arteries	$6.75 \pm 0.2$ (3)	$5.95$ (1)	—	$5.96 \pm 0.8$
<i>Squalus</i>				
Spleen	$6.76 \pm 0.06$ (30)	$6.11 \pm 0.09$ (14)	$5.84 \pm 0.11$ (10)	$4.97 \pm 0.31$
Arteries	$6.29 \pm 0.17$ (6)	$5.37 \pm 0.13$ (14)	$6.06 \pm 0.17$ (5)	$6.63 \pm 0.21$

— = value calculated from only 5 dose-response curves out of 19 expts. (see text).

**Cholinergic drugs** Acetylcholine contracted the artery strips from both *Scyliorhinus* and *Squalus* (Table I Fig. 6). Spleen strips from *Scyliorhinus* did not visibly respond to acetylcholine  $10^{-7}$ – $10^{-8}$  M while *Squalus* spleen strips usually contracted both to acetylcholine (Table I Fig. 6) and carbacholine ( $pD_5 = 6.17 \pm 0.18$ ,  $n = 4$ ). The contraction produced by acetylcholine, however, was often weak and irregular and in only 5 expts. out of 19 cumulative dose-response curves were obtained. The results from these 5 expts. are presented in Table I and Fig. 6.

#### Experiments with perfused spleen

A perfusion pressure of 25–30 cm H<sub>2</sub>O produced an initial flow rate of 0.6–0.7 ml (in *Scyliorhinus*) or 1.0–2.5 ml/min (*Squalus*) respectively through the spleen. Injection of appropriate agonists into the perfusion stream in single doses produced either constriction or dilatation of the spleen. The constriction was manifested as a decrease of the flow on the inflow side, and a biphasic response due to expulsion of fluid from the spleen followed by a decrease in flow rate on the outflow side (Fig. 2 A, 3 A and 3 B). The dilatation was detected as a simple increase in flow on both the inflow and outflow sides (Fig. 2 C, 2 D and 3 C).

TABLE II  $pA_5$  values for the effect of phentolamine on noradrenaline dose-response curves from *Scyliorhinus canicula* and *Squalus acanthias* spleen and artery preparations. Numbers within parentheses indicate number of experiments.

	$pA_5$	$pA_{15}$		Dose range
<i>Scyliorhinus</i>				
Perfused spleen	6.7	5.7	(3)	$10^{-7}$ – $3 \cdot 10^{-6}$ M
Spleen strips	5.9	5.0	(3)	$10^{-6}$ – $10^{-4}$ M
<i>Squalus</i>				
Perfused spleen	7.5	6.6	(4)	$10^{-7}$ – $10^{-6}$ M
Spleen strips	6.7	5.7	(2)	$10^{-6}$ – $10^{-4}$ M
Artery strips	5.9	4.8	(2)	$10^{-6}$ – $10^{-4}$ M

EX-  
NO-  
22

7r

21

22

23

24

25

26

27

28

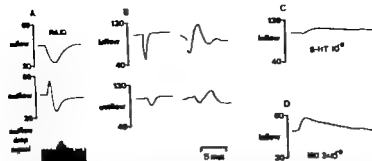
29

30

31

32

33



Effects of drugs and nerve stimulation on the inflow and outflow of perfusion fluid in the dogfish spleen. **A. *Squalius*.** The inflow rate decreases in response to noradrenaline, while the outflow rate increases due to expulsion of fluid from the spleen and then decreases during splenic constriction. The constriction erythrocytes are expelled into the venous effluent. The resulting increase in optical density of the venous effluent is detected by the dropcounter and recorded as an increased amplitude of the drop signal. **B. *Squalius*.** Effects of nerve stimulation with 15 Hz, 0.5 ms duration and 8V for 30 sec. Inflow flow decreases on both the inflow and outflow sides (left), or complex response with initial increase due to expulsion of fluid, period of spleno-constriction and period of splenodilation (right). The latter response was seen in few specimens only. **C. *Squalius*.** Splenodilation produced by 8-hydroxytryptamine. **D. *Squalius*.** Low doses of 8-hydroxytryptamine produce spleno-dilation as shown in A, while higher doses give responses resembling those produced by noradrenaline. Vertical axis refers to the flow rates in drops per minute. All doses are expressed in moles.

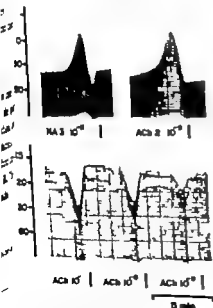


Fig. 3

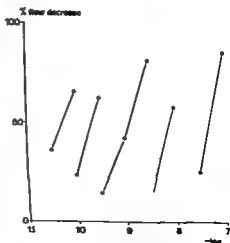


Fig. 4

**3. *Squalius*.** Effects of noradrenaline and acetylcholine on the outflow rate. **A** and **B** show the effects between the drugs on the noradrenaline causes an initial expulsion of fluid from the spleen. **C** shows the dilatory effect of acetylcholine occasionally seen. In this case phentolamine ( $10^{-6}$  M) was used in the perfusion fluid. Note that with this drop recorder an increase in vertical size of the signal means decrease in flow rate, and that the recordings go from right to left.

**4. Parallel shift in the dose-response curve for noradrenaline on the *Squalius* perfused spleen by phentolamine. From left to right: control, phentolamine  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$  and  $3 \times 10^{-6}$  M.**

TABLE III Affinity ( $pD_{50}$ ) values for the different adrenergic agonists tested on perfused spleen preparations from *Scyliorhinus canicula* and *Squalus acanthias*. The values are given as means. Numbers within parenthesis indicate number of experiments.

	Adrenaline	Noradrenaline	Phenylephrine	Isoprenaline
<i>Scyliorhinus</i>	$10.97 \pm 0.31$ (4)	$10.71 \pm 0.22$ (5)	$10.08 \pm 0.54$ (4)	$5.84 \pm 0.24$
<i>Squalus</i>	$9.96 \pm 0.14$ (10)	$9.48 \pm 0.10$ (13)	$7.86 \pm 0.38$ (5)	<5 (9)

At the beginning of each experiment the splenic constriction was accompanied by a release of erythrocytes into the venous effluent, this effect being most pronounced in *S. canicula*. The release of red blood cells is clearly seen as a reddening of the venous effluent. The resulting increase in optical density of the perfusion fluid is often detected as an increase in the amplitude of the outflow drop signal from the photocell (Fig. 2 A).

**Adrenergic drugs** A dose-dependent constriction of the spleen of both species was produced by adrenaline, noradrenaline and phenylephrine (Table III, Fig. 2 A and 3 A), both alone or in lower doses (up to  $3 \cdot 10^{-6}$  mol) produced dilatation of the spleen (Fig. 2 B), dilatation being most pronounced in *Scyliorhinus* and more inconsistent in *Squalus*. Higher doses of isoprenaline caused splenic constriction in both species in a manner resembling other catecholamines and phenylephrine. Dilatation was also produced by 5-hydroxytryptamine ( $10^{-6}$ – $10^{-8}$  mol) in both species (Fig. 2 C), and by salbutamol ( $10^{-6}$ – $10^{-4}$  mol) in *Scyliorhinus*.

Phentolamine competitively antagonized (Fig. 4) the constrictory effects of noradrenaline in both species (Table II). In *Scyliorhinus* a weak dilatation could be seen with noradrenaline after phentolamine ( $10^{-6}$  M).

**Cholinergic drugs** The effects of acetylcholine were in both species very inconsistent. The elucidation of the mechanism of action of this drug is further complicated by the fact that desensitization rapidly occurred during the experiment. The desensitization appeared to be manifested in two phases, one rapid initial phase and one second much slower phase during which some tentative conclusions of drug effects could be made.

In *Scyliorhinus* the most usual response to acetylcholine was a constriction of the spleen.

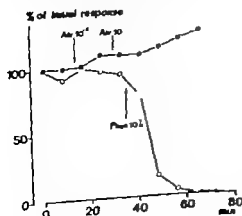
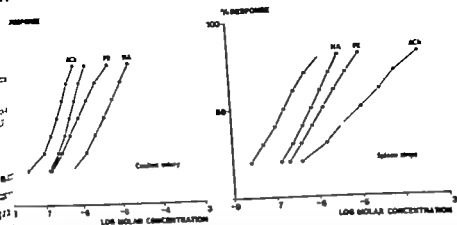


Fig. 5 Effects of atropine (●) or phentolamine (○) reducing the splenic constriction caused by electrical stimulation of the nerves of *Squalus* for 30 min with 15 Hz with pulses of 0.5 ms duration and  $10^{-4}$  M.





6 Dose-response curves in the interval 20–80% of maximal responses for each drug on isolated spleen (right) and coeliac artery strips (left) from *Squalus*. The curves show the mean individual sensitivity (mean and S.E. 1961). A, adrenaline; NA, noradrenaline; PE, phenylephrine; ACh, acetylcholine. It should be noted that the dose-response curve for acetylcholine on the spleen strips is based only on results from one experiment. No corrections for different maximum responses (intrinsic activity) has been made. For number of experiments on each drug consult Table 1.

within the range 8–10), but the initial flow increase on the outflow side due to expulsion of fluid during the start of the constriction was not present unless high doses of acetylcholine were applied (Fig. 3 B). Phenolamine ( $10^{-6}$  M) abolished the initial flow increase caused by high doses of acetylcholine, and in two cases the constrictory response to acetylcholine was changed to a dilatation (Fig. 3 C). Occasionally acetylcholine exerted only dilatatory effects about previous addition of alpha adrenoceptor blockers. Atropine ( $10^{-4}$ – $2 \cdot 10^{-4}$  M) completely blocked those constrictory effects of acetylcholine which are not subject to the rapid desensitization, and in two cases also the dilatatory responses, although the effect was impossible to quantify due to desensitization.

In *Squalus* the normal response to acetylcholine was a constriction mimicking that caused by adrenaline. In one case a slight dilatation was produced by acetylcholine ( $10^{-4}$  mol), and in 3 expts. no response at all could be obtained. Phenolamine ( $10^{-6}$ – $10^{-4}$  M) reduced, although never abolished, the effects of  $10^{-6}$ – $10^{-4}$  mol of acetylcholine during the slower phase of desensitization. Mecamylamine ( $10^{-6}$  M) was without detectable effects on the acetylcholine response.

**Nerve stimulation.** Electrical stimulation of the vascular bundles to the spleen suggests that few if any sympathetic nerves pass to the spleen in *Squalus*. In 3 expts. out of 13 a very slight flow decrease was recorded in response to electrical stimulation with 20 Hz, 0.5–1.0 ms duration and 3–18 V for 30–60 s. The responses were too small and inconsistent to allow further analysis.

In *Squalus*, however, significant and reproducible changes in perfusion flow through the spleen occurred during stimulation of the splenic nerves following the lino-gastric artery (Fig. 2 B). Stimulation carried out with 0.5–1.0 ms duration, 10–20 Hz and 3–12 V for 30 s with 8 min intervals gave reproducible responses for at least 2 h. The flow change during

TABLE III. Affinity ( $pD_5$ ) values for the different adrenergic agonists tested on perfused spleen preparations from *Scyllorhinus canicula* and *Squalus acanthias*. The values are given as mean. Numbers within parenthesis indicate number of experiments.

	Adrenaline	Noradrenaline	Phenylephrine	Isoprenaline
<i>Scyllorhinus</i>	$10.97 \pm 0.31$ (4)	$10.71 \pm 0.22$ (5)	$10.08 \pm 0.54$ (4)	$5.94 \pm 0.42$ (1)
<i>Squalus</i>	$9.96 \pm 0.14$ (10)	$9.48 \pm 0.10$ (13)	$7.86 \pm 0.38$ (5)	<5 (4)

At the beginning of each experiment the splenic constriction was accompanied by a release of erythrocytes into the venous effluent, this effect being most pronounced in *S. canicula*. The release of red blood cells is clearly seen as a reddening of the venous effluent. The resulting increase in optical density of the perfusion fluid is often detected as an increase in the amplitude of the outflow drop signal from the photocell (Fig. 2 A).

**Adrenergic drugs.** A dose-dependent constriction of the spleen of both species was produced by adrenaline, noradrenaline and phenylephrine (Table III, Fig. 2 A and 3 A). Isoprenaline in lower doses (up to  $3 \times 10^{-8}$  mol) produced dilatation of the spleen (Fig. 2 B), dilatation being most pronounced in *Scyllorhinus* and more inconsistent in *Squalus*. Higher doses of isoprenaline caused splenic constriction in both species in a manner resembling other catecholamines and phenylephrine. Dilatation was also produced by 5-hydroxytryptamine ( $10^{-8}$ – $10^{-6}$  mol) in both species (Fig. 2 C), and by salbutamol ( $10^{-8}$ – $10^{-6}$  mol) in *Scyllorhinus*.

Phentolamine competitively antagonized (Fig. 4) the constrictory effects of noradrenaline in both species (Table II). In *Scyllorhinus* a weak dilatation could be seen with noradrenaline after phentolamine ( $10^{-6}$  M).

**Cholinergic drugs.** The effects of acetylcholine were in both species very inconsistent. The elucidation of the mechanism of action of this drug is further complicated by the fact that desensitization rapidly occurred during the experiment. The desensitization appeared to be manifested in two phases, one rapid initial phase and one second much slower phase. In which some tentative conclusions of drug effects could be made.

In *Scyllorhinus* the most usual response to acetylcholine was a constriction of the

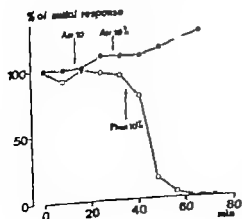


Fig. 3 Effects of tropine (●) or phentolamine (○) on reducing the splenic constriction caused by electrical stimulation of the nerves of *Squalus* for 30 s every 4 s at 15 Hz with pulses of 0.5 ms duration and 10 V.

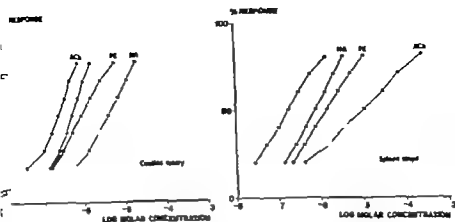


Fig. 16. Dose-response curves in the interval 20–80% of maximal response for each drug on isolated spleen (right) and coeliac artery strips (left) from *Spizella*. The curves show the mean individual sensitivity (see also Sano and Sano, 1961). A, adrenaline; NA, noradrenaline; PE, piperylphepine; ACh, acetylcholine. It should be noted that the dose-response curve for acetylcholine on the spleen strips is based only on results from strips where proper dose-response curves could be obtained. No corrections for different maximum means (intrinsic activity) has been made. For number of experiments on each drug consult Table I.

within the range  $8-10$ , but the initial flow increase on the outflow side due to expulsion fluid during the start of the constriction was not present unless high doses of acetylcholine were applied (Fig. 3 B). Phentolamine ( $10^{-6}$  M) abolished the initial flow increase caused by high doses of acetylcholine, and in two cases the constrictory response to acetylcholine was turned to a dilatation (Fig. 3 C). Occasionally acetylcholine exerted only dilatatory effects without previous addition of  $\alpha$  adrenoceptor blockers. Atropine ( $10^{-6}-2 \cdot 10^{-6}$  M) completely blocked these constrictory effects of acetylcholine which are not subject to the most rapid desensitization, and in two cases also the dilatatory response, although the effect was impossible to quantify due to desensitization.

In *Spizella* the normal response to acetylcholine was a constriction mimicking that caused by adrenaline. In one case a slight dilatation was produced by acetylcholine ( $10^{-6}$  mol), and in 13 expts. no response at all could be obtained. Phentolamine ( $10^{-7}-10^{-6}$  M) reduced, although never abolished, the effects of  $10^{-6}-10^{-4}$  mol of acetylcholine during the slower phase of desensitization. Mecamylamine ( $10^{-6}$  M) was without detectable effects on the acetylcholine response.

**Acetylcholine stimulation.** Electrical stimulation of the vascular bundles to the spleen suggests that few if any sympathetic nerves pass to the spleen in *Scylorhynchus*. In 3 expts. out of 13 a very slight flow decrease was recorded in response to electrical stimulation with 20 Hz, 0.5–1.0 ms duration and 3–18 V for 30–60 s. The responses were too small and inconsistent to allow further analyses.

In *Spizella*, however, significant and reproducible changes in perfusion flow through the spleen occurred during stimulation of the splenic nerves following the lienogastro-artery (Fig. 3 B). Stimulation carried out with 0.5–1.0 ms duration, 10–20 Hz and 3–12 V for 30 s with 3 min intervals gave reproducible responses for at least 2 h. The flow change during

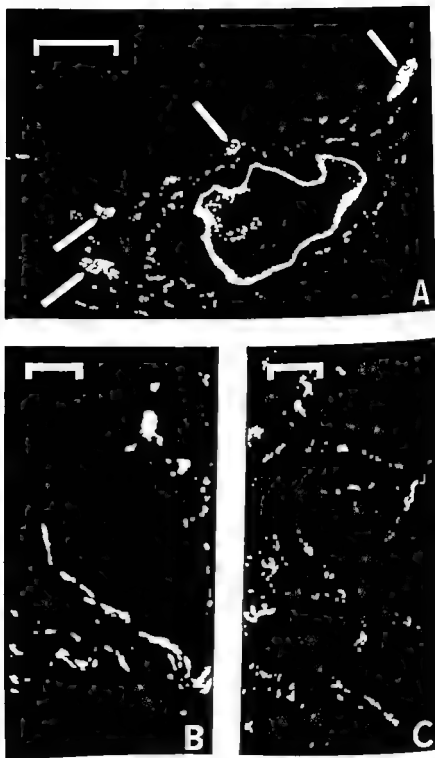


Fig. 7 *Symplocos beno-graine* artery. Formaldehyde treatment for 1 h. A. Diagonal section through artery wall showing a well developed innervation by fluorescent f. bras. At arrows large bundles of fluorescent fibres following the vessel. Not the strongly autofluorescent inner elastic membranes. Calibration 400  $\mu\text{m}$ . B. Bundle of varicose f. bras. the artery wall. Calibration 25  $\mu\text{m}$ . C. Longitudinal section through the artery wall. Mesh-work of smooth and terminal fluorescent f. bras. Background fluorescence due to autofluorescent connective tissue. Calibration 100  $\mu\text{m}$ .



Fig. 8. Squid. 8) sympathetic ganglion (A, B) and spleen (C). Formaldehyde treatment for 1 h. A. Section one of the anterior sympathetic ganglia, the gastric ganglion, showing ganglion cells surrounded by nerveous fibres and strongly fluorescent chromaffin tissue. The right bundle of nerve fibres leaves the ganglion. Calibration 250  $\mu$ m. B. Weakly fluorescent ganglion cells surrounded by brightly fluorescent nerve. Calibration 50  $\mu$ m. C. Fluorescent nerve fibres in the spleen of an animal pre-injected with noradrenaline. Background fluorescence due to yellow and green cellular autofluorescence. Calibration 50  $\mu$ m.

stimulation is sometimes polyphasic in both the inflow and outflow sides, including a period of spleno-dilatation (Fig. 2 B). This splenodilatation occurs whether or not the denervation stimulation is continued, and is thus not due to any "rebound" phenomenon (Campbell & Burnstock 1968).

The splenic constriction produced by nerve stimulation could be rapidly blocked by phentolamine ( $10^{-6}$  M) but was unaffected or slightly enhanced by atropine ( $10^{-4}$ – $10^{-5}$  M) (Fig. 5). Mecamylamine ( $10^{-6}$  M) lacked detectable effects. Where a dilatation followed constriction during nerve stimulation this was also invariably abolished by phentolamine ( $10^{-6}$  M). Nicotine caused a weak rapidly desensitized constriction in the *Squalius* spleen.

### *Fluorescent histochemistry*

In the coeliac and lieno-gastric arteries characteristically bluish-green brightly fluorescent nerve fibres could be seen after treatment with formaldehyde vapour for 1 h (Fig. 7 A, B). Large bundles of smooth fluorescent fibres could also be seen following both the lieno-gastric (Fig. 7 A) and the coeliac arteries. Bright autofluorescence was seen in the inner cell membrane (Fig. 7 A).

Treatment for 1–3 h in formaldehyde vapour of spleen preparations from nonadrenalin-preinjected animals produced but few fluorescent terminals in the spleen tissue except close to the small arteries (Fig. 8). The presence of strong yellow or green autofluorescence would obscure nerve fibres in most parts of the spleen.

In sympathetic ganglia weakly but specifically fluorescent ganglion cells with surmountable terminals could be seen after 1 h formaldehyde treatment (Fig. 8) together with extensive bright clusters of chromaffin cells (Fig. 8 A).

### Discussion

Contrary to earlier reports (Opdyke and Opdyke 1971) the dogfish spleen in our experiments is able to release erythrocytes when stimulated. The release of red blood cells can clearly be observed to be smaller than in the cod (Nilsson and Grove 1974), and may therefore represent a less developed stage in this respect.

The presence of alpha adrenoceptors, in both the spleen and arteries, mediating contraction of the tissues is demonstrated in both species from the order of potency of the adrenoceptor agonists and the  $pA$  values for phentolamine. It is interesting to note that in *Squalius* phentolamine has a higher  $pD$  than noradrenaline on the arteries, which may in part be due to the simultaneous noradrenaline and adrenaline effects simultaneously on beta adrenoceptors. The parallel shift of the noradrenaline dose response curve produced by phentolamine in all preparations tested, indicates that this drug acts by competitively blocking the alpha adrenoceptors of the smooth muscles.

The unpredictable nature of the dilatation produced by isoprenaline did not allow experiments with beta adrenoceptor antagonists, so we can only suggest that it is due to a beta adrenoceptor mechanism since noradrenaline (after phentolamine) and salbutamol also had weak dilatatory actions.

The variability of the muscarinic reaction to acetylcholine may depend on simultaneous

ions of trabecular, capsular and intrinsic blood vessel smooth muscle cells. It is possible, ple, for contraction of certain trabeculae to enlarge the local blood space. In the ace of detailed knowledge of the sites of action of acetylcholine, particularly in *Scyllorhinus* which has little splenic innervation, an explanation of the observed acetylcholine must await a more detailed study of blood flow through the spleen of these fish. he part of the acetylcholine response which is subject to the most rapid desensitization in part be due to release of catecholamine (in *Squalus*). This may be noradrenaline, h is the dominating catecholamine in several organs in this animal (Euler and Flägel 1).

oth the constrictory and dilatatory effects in the polyphasic response to electrical stimula- sometimes seen in *Squalus* can be rapidly blocked by phentolamine in relatively low oncentrations, and this may be taken to indicate that the nerve effects are ultimately media- by an adrenergic transmitter. Fluorescent histochemistry shows few adrenergic nerve inals in the spleen and the innervation may regulate mainly the vessels. It is also possible t the bright autofluorescence obscures specifically fluorescent nerve terminals on the spleen cle cells.

n *Scyllorhinus* the innervation of the spleen seems to be poorly developed as judged from it or absent responses to electrical stimulation of the vascular bundle. Regulation of the tes and release of erythrocytes in this species may largely depend on the levels of circu- adrenaline and noradrenaline in the blood. Marrod (1969) found in the 'normal' *Scyllorhinus* blood plasma 2.4  $\mu\text{g}$  adrenaline and 4.8  $\mu\text{g}$  noradrenaline per 100 ml plasma, which in nimals kept in air for 30 min was increased to 8.5 and 13.8  $\mu\text{g}$  per 100 ml plasma respec- ily. These values compare closely with the observed concentration ranges over which effects of adrenaline and noradrenaline occur in isolated artery and spleen strips. (In *Scyllorhinus*  $\text{ED}_{50}$  for spleen strips 11.8 and 42.3  $\mu\text{g}/100\text{ ml}$  and  $\text{ED}_{50}$  for artery strips 3.6 d 16.9  $\mu\text{g}/100\text{ ml}$  for adrenaline and noradrenaline respectively.) Such changes in the catecholamine concentration in the blood plasma may therefore control both the spleen and arteries. Davies and Rankin (1973) similarly concluded that circulating catecholamines ay an important role in controlling the branchial circulation in *Scyllorhinus*. By the same gement, circulating catecholamines will reinforce the sympathetic innervation of the yster spleen.

he work was supported by the Swedish Natural Science Research Council and the Kurt and Alice Wallen- erg Travelling Fund.

## References

- ARON, E. J. and A. M. SONNEN, Analysis of the action of drugs and drug combinations. In: *Quantitative methods in pharmacology*. Ed. by H. de Jongh, North Holland Publishing Corp., Amsterdam 1961. 284-311.
- KNY, B. G. and S. A. ORSICO, Antagonism of acetylcholine by adrenaline antagonists. *Brit. J. Pharmacol.* 1963 20 528-533.
- KNY, V. De l'influence de l'adrénaline et de l'angiotensine sur le nombre des hématocytes dans le sang circulant des poissons osseux. *J. Physiol. (Paris)* 1929 27 735-740.
- ARWELL, G. and G. BLUMBERG, Comparative physiology of gastrointestinal motility. In: *Handbook of Physiology*, Vol. IV Section 6, Alimentary canal: Motility. Ed. by C. P. Code, American Physiological Society Washington DC. 1968. 2213-2246.

stimulation is sometimes polyphasic in both the inflow and outflow sides, including a period of spleno-dilatation (Fig. 2 B). This splenodilatation occurs whether or not the denervation stimulation is continued and is thus not due to any "rebound" phenomenon (Campbell & Burnstock 1968).

The splenic constriction produced by nerve stimulation could be rapidly blocked by phentolamine ( $10^{-5}$  M) but was unaffected or slightly enhanced by atropine ( $10^{-4}$  M) (Fig. 5). Mecamylamine ( $10^{-6}$  M) lacked detectable effects. Where a dilatation followed constriction during nerve stimulation, this was also invariably abolished by phentolamine ( $10^{-5}$  M). Nicotine caused a weak rapidly desensitized constriction in the *Squalus* spleen.

#### Fluorescent histochemistry

In the coeliac and ileo-gastric arteries characteristically bluish-green brightly fluorescent nerve fibres could be seen after treatment with formaldehyde vapour for 1 h (Fig. 7 A, B). Large bundles of smooth fluorescent fibres could also be seen following both the ileo-gastric (Fig. 7 A) and the coeliac arteries. Bright autofluorescence was seen in the inner cell membrane (Fig. 7 A).

Treatment for 1-3 h in formaldehyde vapour of spleen preparations from normal noradrenaline preinjected animals produced but few fluorescent terminals in the spleen, except close to the small arteries (Fig. 8). The presence of strong yellow or green autofluorescence would obscure nerve fibres in most parts of the spleen.

In sympathetic ganglia weakly but specifically fluorescent ganglion cells with somatic terminals could be seen after 1 h formaldehyde treatment (Fig. 8) together with extremely bright clusters of chromaffin cells (Fig. 8 A).

#### Discussion

Contrary to earlier reports (Opdyke and Opdyke 1971) the dogfish spleen in our experiments is able to release erythrocytes when stimulated. The release of red blood cells can clearly be observed to be smaller than in the cod (Nilsson and Grove 1974) and may therefore represent a less developed stage in this respect.

The presence of alpha adrenoceptors, in both the spleen and arteries, mediating contraction of the tissues is demonstrated in both species from the order of potency of the adrenoceptor agonists and the pA values for phentolamine. It is interesting to note that in *Squalus* phentolamine has a higher pD than noradrenaline on the arteries, which may in part be due to noradrenaline and adrenaline effects simultaneously on beta adrenoceptors. The parallel shift of the noradrenaline dose-response curve produced by phentolamine in all preparations tested indicates that this drug acts by competitively blocking the alpha adrenoceptors of the smooth muscles.

The unpredictable nature of the dilatation produced by isoprenaline did not allow proper tests with beta adrenoceptor antagonists, so we can only suggest that it is due to a beta adrenoceptor mechanism since noradrenaline (after phentolamine) and salbutamol also had weak dilatatory actions.

The variability of the muscarinic reaction to acetylcholine may depend on simultaneous



## Studies of Some Twitch and Fatigue Properties of Different Motor Unit Types in the Ankle Muscles of the Adult Cat

By

C. HAMMARBERG and J-O KELLERTH

Received 27 March 1975

### Abstract

**HAMMARBERG, C. and J-O KELLERTH.** Studies of some twitch and fatigue properties of different motor unit types in the ankle muscles of the adult cat. *Acta physiol. scand.* 1975 **95**: 231-242.

Electrical responses of motor units in the gastrocnemius, soleus and pretibial flexor muscles of adult cats were elicited by microstimulation of motoneurons. The motor units were classified into types FF and S (Burke *et al.* 1971) and their responses to the same stimulation patterns as those used in vivo investigation of whole muscles (Hammarberg and Kellerth 1975a) were studied. The duration of membrane afterhyperpolarization was short in both the fast twitch FF and FR units. It was longer in some S units than in the S units of the pale muscles. Twitch time-to-peak was less than 30 ms in FF and FR units, but exceeded 40 ms in the S units. Soleus S units were slower than S units of the pale soleus. Facilitation was observed in the gastrocnemius units, but not in the soleus S units. A short rest period followed extensive work of the FF and FR types to regain some contractile strength. This was less evident in the S units which, on the other hand, were extremely resistant to fatigue. Differences in response time between corresponding motor unit types of the flexor and extensor muscles were observed. A few track units were identified in the slow soleus muscle.

In a previous investigation (Hammarberg and Kellerth 1975a) the postnatal development of certain twitch and fatigue properties of the medial gastrocnemius, soleus and anterior tibial muscles of the cat was studied. In order to better understand the postnatal changes in motor function it was necessary to extend the investigation to the level of single motor units as well, since the performance of whole muscles reflects the summated activity of a large number of motor units which are known to display differing functional properties (Devanandan *et al.* 1965, Henneman and Olson 1965, McPhedran *et al.* 1965, Wurster *et al.* 1965, Burke *et al.* 1971, 1973, 1974, Olson and Swett 1971, Gossow *et al.* 1972, Mather *et al.* 1972, Bagust *et al.* 1973, Bagust 1974, Stephens and Stuart 1974).

In the present study different types of motor units of the ankle muscles of the adult cat have been investigated with respect to their responses to the same functional tests as those applied in the previous study of whole muscles (Hammarberg and Kellerth 1975a). In addition to the twitch and fatigue properties of the muscle fibres, the duration of the post-spike after

- DAVIES, D. T. and J. C. RANKIN, Adrenergic receptors and vascular responses in catecholamine-depleted dogfish gills. *Comp. gen. Pharmacol.* 1973. 4: 139-148.
- EULER, U. S. and R. FÄNGE, Catecholamines in nerves and organs of *Myxine platensis*, *Squalus acanthias* and *Gadus callarias*. *Gen. comp. Endocr.* 1961. 1: 191-194.
- FALCK, B. and CH. ÖWMAN, A detailed methodological description of the fluorescence microchemical demonstration of biogenic amines. *Acta Univ. Lund.* 1965. Sect. II No 7.
- HOLMES, W. N. and E. M. DONALDSON, The body compartments and distribution of catecholamines. In: *Physiology* Ed. by W. S. Hoar and D. J. Randall, Academic Press, New York and London (1967) 1-89.
- HOLMÖREN, S. and S. NILSSON, Effects of some adrenergic and cholinergic drugs on isolated spleens from the cod, *Gadus morhua*. *Eur. J. Pharmacol.* 1975. 32: 163-169.
- MAZEAUD, M., Influence de stress sur les teneurs en catecholamines du plasma et des corps splanchniques chez un Selacien, la Rougette (*Scorpaenopsis diabolus* L.). *C. R. Soc. Biol. (Paris)* 1969. 163: 2262-2266.
- NILSSON, S. and D. J. GROVE, Adrenergic and cholinergic innervation of the spleen of the cod, *Gadus morhua*. *Eur. J. Pharmacol.* 1974. 28: 135-141.
- OPDYKE, D. F. and N. E. OPDYKE, Splenic responses to stimulation in *Squalus acanthias*. *Amer. J. Physiol.* 1971. 221: 623-625.
- ROSSUM, J. M. VAN, Cumulative dose-response curves. II. Technique for the making of dose-response curves in isolated organs and the evaluation of drug parameters. *Arch. int. Pharmacodyn.* 1961. 129: 299-330.
- VAIREL, J. Action de l'adrénaline et de l'acétylcholine sur la rate. *J. Physiol. Path. exp.* 1933. 31: C.

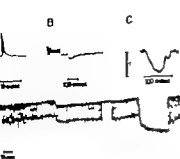


Fig. 1

Motor unit of the S type belonging to the MG muscle. *A* Motoneurone action potential. *B* Neurone AHP following an action potential elicited by intracellular stimulation. *C* Twitch contraction of the muscle unit. *D* Contractile responses of the muscle unit to the sequentially occurring "fatigue" and "tetanus" tests. In *C* and *D* as well as in the following figures, an increase in contractile force is signified by downward deflection in the myogram.

Motor unit of the S type belonging to the SOL muscle. *A* Motoneurone action potential. *B* Neurone AHP following an action potential elicited by intracellular stimulation. *C* Twitch contraction of the muscle unit. *D* Contractile responses of the muscle unit to the sequentially occurring "fatigue" and "tetanus" tests.

Fig. 2

It cannot be compared directly with those of Burke *et al* (1971, 1973), however, since the latter authors had maximal post-tetanic potentiation in their units prior to the "fatigue test" with the result that the maximal tension output always occurred during the first contraction of the sequence. In the present study maximal responses sometimes did not occur until 15–20 s after the onset of the "fatigue test". Burke *et al* (1971, 1973, 1974) also stimulated their motor units with pulse trains of 2 s duration, each with an interval corresponding to 125% of the contraction time of the unit. This mode of stimulation produced maximal tetanus and was used by Burke and his associates to separate the two fatigue resistant unit types "R" (fast contracting, fatigue resistant) and "S" (slow contracting, fatigue resistant). This so-called "5 test" was used only in the earlier part of the present study since it was discovered that FR and S units could also be separated by means of the "passive" and "tetanus tests".

## Results

The results presented here were obtained from 116 motor units belonging to the MG (52 units), LG (36 units), SOL (17 units), TA (3 units) and EDL (8 units) muscles of adult cats. *S units of triceps surae* Fig. 1 and 2 show typical examples of the behaviour of S units of the MG, LG and SOL muscles, respectively during the various tests. *A* of Fig. 1 and 2 shows the action potentials, while *B* illustrates at a slower sweep speed the afterhyperpolarizations (AHP). Generally the AHP's of the SOL motoneurons were of longer duration than those of the S units of the MG+LG muscles ( $p < 0.002$ , Table 1). *C* of Fig. 1 and 2 shows the twitch contractions elicited by intracellular stimulation of the motoneurons. The mean value for twitch contraction time (time-to-peak) was considerably greater for the SOL S units than for the S units of gastrocnemius ( $p < 0.001$ ) although there was some overlap of values between the two populations (Fig. 3 and Table I). Also the mean values for twitch half-relaxation time (HRT) were found to be different for the two groups of S units ( $p < 0.05$ , Table I).

*D* of Fig. 1 and 2 illustrates the contractile responses of the S units of the gastrocnemius

hyperpolarization (AHP) of the innervating motoneurons has also been investigated. This parameter had earlier been shown to differ between motoneurons supplying fast and slow twitch muscle fibres, respectively (Eccles *et al.* 1958, Kuno 1959). The corresponding properties of immature motor units at various stages of postnatal development are described in a following report (Hammarberg and Kellerth 1975 b).

### Methods

Adult cats were anaesthetized with an intraperitoneal injection of Nembutal (40 mg/kg; all amounts of anaesthetics, if needed, were administered during the course of the experiment through a catheter inserted in the left femoral vein. The medial gastrocnemius (MG), lateral gastrocnemius (LG) (SOL), anterior tibial (TA) and extensor digitorum longus (EDL) muscles of the right hind leg were carefully dissected free without damage to their blood or nerve supplies. The corresponding tendons were cut and arranged for separate attachment to a strain gauge myograph through short brided sutures (pliance 0.05 mm/kg cm). The motor nerves to the MG, LG + SOL and TA + EDL muscles were cut and surrounded by glass tubes and mounted intact on stimulating electrodes. The remaining portions of the peroneal and common peroneal nerves were cut or crushed.

After a lumbosacral laminectomy the ipsilateral dorsal roots L6-S2 were transected. The animal was placed in a rigid steel frame and immobilized along the spinal column, pelvic girdle and right hind leg. Exposed tissues were covered with paraffin oil. The rectal and muscle temperatures were maintained at 35-38°C by means of infrared light. During the course of the experiment the animals were allowed to breathe spontaneously through a tracheal cannula, except in the few cases where it was necessary to perform a pneumothorax in order to decrease respiratory movements. In such cases artificial ventilation was used. In all animals expired CO<sub>2</sub> was monitored continuously since hyper- or hypoventilation have been shown to markedly influence the fatigue properties of muscles (Hammarberg and Kellerth 1975 a). Single motor units were impaled with glass micropipettes with tip diameters of 1-2 µm. The micropipettes were filled either with 4M potassium citrate (ohmic resistance 3-8 MΩ), or more often with a 5% solution of the dye Procion Yellow (in distilled water) (ohmic resistance 10-15 MΩ), since one of the aims of the present experiments was to stain physiologically intact motoneurons for later histological analysis. Following impalement each motoneuron was identified by antidromic stimulation of the muscle nerves. Its appropriate muscle was then connected to the strain gauge, and contractile responses of the muscle fibres innervated by it were elicited by intracellular stimulation of the motoneurons (depolarizing pulses of 2 ms duration). For each motor unit the passive stretch of the muscle was adjusted to give maximal twitch responses. In all cases, however, the amount of passive stretch was less than that corresponding to 90° dorsiflexion of the ankle. During the course of the experiment, regular checks were made of the twitch tensions of the whole muscles. If a reduction in mechanical activities of the motor units was observed, the experiment was terminated. The electrical activities of the motor units were displayed on an oscilloscope and by a de-coupled power

### Testing procedures

Immediately following the impalement of a motoneuron, its membrane potential and antidromic action potential were recorded. However, since these measurements were probably often distorted due to high ohmic resistances, they were used only for continuous monitoring of cell condition during the time of impalement. The afterhyperpolarization (AHP) following action potentials evoked by intracellular stimulation was studied.

The contractile responses of muscle fibres belonging to single motor units were studied both under single pulse stimulation and during the different modes of repetitive stimulation described in the present study on whole muscles, i.e. the "fatigue", "pause" and "tetanus" tests (Hammarberg and Kellerth 1975). In the latter investigation the pause test was initiated both before and after the "tetanus test". Of the first of these two "pause tests" has been considered in the present study since erroneous results due to cell deterioration are more liable to appear with prolonged periods of microelectrode impalement. The "fatigue index" of a unit was calculated as the ratio of the amplitude of the 120th contraction of the "fatigue test" to that of the first contraction of the sequence. The "fatigue indices" of the present study



Fig. 3

Fig. 4

I. Motor unit of the FR type belonging to the LG muscle. A. Motor unit action potential. B. monophasic AHP following an action potential elicited by intracellular stimulation. C. Twitch contraction of the muscle unit. D. Contractile responses of the muscle unit to the sequentially occurring "fatigue" and "tetanus tests". E. Contractile response to unfused tetanic stimulation (the "sag test").

II. Motor unit of the FR type belonging to the MG muscle. A. Motor unit action potential. B. monophasic AHP following an action potential elicited by intracellular stimulation. C. Twitch contraction of the muscle unit. D. Contractile responses of the muscle unit to the sequentially occurring "fatigue" and "tetanus tests".

#### units of triceps surae

Fig. 3 shows response pattern typical of FR units. In general the AHP's of these units are of shorter duration than those of the S type ( $p < 0.01$ , Fig. 8, Table I). The mean time to peak of twitch contractions was also considerably less for FR units than for the S units ( $p < 0.001$ ), and in no case did the contraction time exceed 30 ms (Fig. 8, Table I). E of Fig. 3 shows the muscle response to unfused tetanus, where the presence of sag further separates the FR units from the S type of units (Burke *et al.* 1971, Prosser and White 1974).

D of Fig. 3 shows contractile responses during the "fatigue" "pause" and "tetanus tests". By definition, the "fatigue index" of an FR unit (Fig. 3) is  $> 0.75$ . In the present study "fatigue indices" up to 1.67 were encountered (see also Stephens and Stuart 1974). In extensor units of the FR type, the 10 s pause resulted in a significant ( $p < 0.001$ ) increase in contractile strength when stimulation was resumed (Fig. 7 Table I). In some cases, the tension developed immediately after the pause even exceeded that produced earlier during the "fatigue test" revealing most likely a potentiating effect of the iterative stimulation (Olson and Sencoff 1971). In contrast to the S units (Fig. 1 D and 2 D), the FR units showed no immediate change in tension output with the onset of this tetanus (Fig. 3 D), the t/p tension ratio was close to 1.0 (Fig. 7 and Table I). However, during the course of the tetanus the contractile tension of typical FR units gradually increased ( $24.4 \pm 29.1\%$ ), and this effect even remained for some time after the iterative stimulation had been resumed (Fig. 3 D). From the responses of the FR and S units it may therefore be concluded that by using the same tests which had previously been applied to study differences in fatigability among whole muscles (Hammarberg and Kellorh 1975 a) it is also possible to separate extensor FR and S units.

TABLE I. A summary of observations of 105 motor units belonging to the triceps surae muscle. For each parameter the mean value  $\pm$  SE has been indicated. The total range of values is given in parentheses. Because of the large relative error introduced when trying to measure small, often negligible, contractile tensions produced by the FF units during the "pause test" the corresponding values have been omitted in the Table.

Motor unit type	Duration of motoneurone AHP (ms)	Contraction time (ms)	HRT (ms)	Fatigue index	Pause effect (%)	1st tetanic tension
FF (n=46)	110 $\pm$ 36 (50-190)	18.3 $\pm$ 3.4 (11-27)	13.1 $\pm$ 4.9 (8-23)	0.07 $\pm$ 0.06 (0.00-0.5)	—	—
FR (n=21)	101 $\pm$ 26 (70-160)	20.9 $\pm$ 4.1 (16-29)	20.5 $\pm$ 5.1 (1-28)	0.97 $\pm$ 0.36 (0.77-1.67)	20.2 $\pm$ 4.1 (15-30)	1.86 $\pm$ 0.18 (1.08-1.8)
S (n=9)	136 $\pm$ 25 (90-190)	49.1 $\pm$ 12.6 (40-75)	53.8 $\pm$ 15.3 (36-80)	1.06 $\pm$ 0.11 (0.98-1.18)	4.0 $\pm$ 3.9 (0-10)	1.13 $\pm$ 0.12 (0.75-1.5)
Soleus S (n=13)	198 $\pm$ 30 (145-250)	75.4 $\pm$ 13.6 (52-95)	77.3 $\pm$ 6.6 (36-110)	0.94 $\pm$ 0.06 (0.85-1.00)	5.5 $\pm$ 9 (0-9)	1.25 $\pm$ 0.14 (1.14-1.3)
Unclassified (n=16)	105 $\pm$ 27 (65-155)	22.1 $\pm$ 4.8 (13-29)	16.6 $\pm$ 5.9 (10-24)	0.55 $\pm$ 0.75 (0-5)	27.8 $\pm$ 14.6 (12-55)	1.00 $\pm$ 0.12

and soleus muscles during prolonged activation. Each record begins with a "tetanus" lasting for 2 min, followed by a 10 s pause. Iterative stimulation was then resumed, but again interrupted after approximately another 30 s by the "tetanus test". The S units were very resistant to fatigue and, by definition, develop at least 75% of their initial contraction after 2 min of the "fatigue test" i.e. they have a "fatigue index"  $> 0.75$  (Burke 1971). In the present study the soleus S units had "fatigue indices" which were smaller than those of the gastrocnemius S units ( $p < 0.05$ , Table I). The changes in contractile tension following the "pause test" were quite insignificant in all cases, even for those S units which had displayed the lowest values for fatigue index.

Immediately with the onset of the "tetanus test" all S units developed a tension which exceeded that of the last contraction of the preceding "fatigue test" by more than 15% (Fig. 1 and 2), i.e. they had tetanus/pretetanus (t/p) tension ratios greater than 1.15 (Fig. 7 and Table I). Furthermore, the S units maintained their tension throughout the period of tetanic stimulation and exhibited no signs of fatigue. Sometimes the S units of MG+LG but not those of SOL, even showed a gradual increase in tension ( $7.4 \pm 10.2\%$ ) during tetanus (Fig. 1). This difference ( $p < 0.05$ ) between the S units of the two muscles can possibly be accounted for by a difference between them in potentiation (Olsson and Sjöström 1971, Burke *et al.* 1974). This possibility is supported by the increased amplitude of contractions immediately following the tetanus of the S units of MG+LG (Fig. 1), as well as by the observed tendency for these units to have a somewhat larger "fatigue index" ( $p < 0.05$ , Table I). In summary the S units of MG+LG and SOL are similar with respect to their responses to the various modes of repetitive stimulation, even though the units of the former muscles seem to be more potentiated, but there are also clear dissimilarities between them in their mean values for contraction time and AHP duration (Fig. 7 and 8, Table

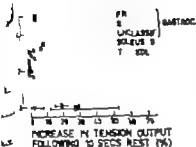


Fig. 7

Graphical illustration of the relation between the t/p tension ratio (ordinate) and the "pause effect" (ms) for extensor and flexor motor units of different types.

Three dimensional plot showing the relationship between the motoneurone AHP (vertical Y-axis), rest time-to-peak (horizontal X-axis) and the "fatigue index" (horizontal Z-axis) for motor units belonging to the SOL, MG, LG, and TA, EDL muscles.

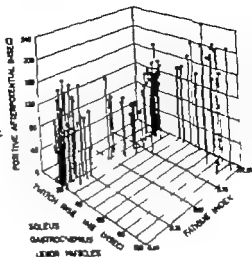


Fig. 8

FF units ( $p < 0.05$ ) and smaller than that of the FR units ( $p < 0.1$ , Table I). With respect to their responses to the "fatigue" and "tetanus tests" the "unclassified" units at 3 times resembled the FR units (Fig. 5), and at other times they more closely resembled FF type of units (Fig. 6). The average decrease in tension during the course of the tetanus test amounted to  $89.7 \pm 9.9\%$ . The pause test usually caused a somewhat recovery in contractile tension in the "unclassified" units than in the FR units (Table I).

For smaller units

For considerable effort only 11 motor units belonging to the TA (3) and EDL (8) muscles were successfully studied in the present investigation. Ten of these units had contraction times varying between 16 and 24 ms (mean value  $20.8 \pm 2.5$  ms) and were assigned to the (3 units), FR (5 units) and "unclassified" (2 units) types on the basis of their responses to the "fatigue test". One fatigue resistant unit with contraction time of 36 ms was classified as type 5 unit, since it did not "sag" (Burke *et al.* 1973). The duration of motoneurone AHP of the fast twitch units varied between 65 and 110 ms (mean value  $89.0 \pm 14.7$  ms), while the AHP duration of the type 5 unit was 155 ms.

Although it is not possible on the basis of the small number of units studied here to draw any definite conclusions, it is our impression that with regard to the responses to repetitive stimulation, the flexor units present a picture which is somewhat different from that of the extensor units (Fig. 7 see also Hammarberg and Kellerth 1975 b). For example, the flexor

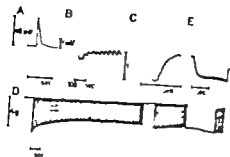


Fig. 5

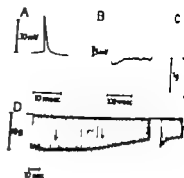


Fig. 6

Fig. 5. Unclassified motor unit belonging to the SOL muscle. A. Motoneuron action potential. B. Motoneuron AHP following an action potential elicited by intracellular stimulation of the muscle unit. C. Twitch contraction. D. Tetanic contraction. Scale bars: 10 mV, 10 ms.

Fig. 6. Unclassified motor unit belonging to the LG muscle. A. Motoneuron action potential. B. Motoneuron AHP following an action potential elicited by intracellular stimulation of the muscle unit. C. Twitch contraction. D. Tetanic contraction. Scale bars: 10 mV, 10 ms.

#### FF units of triceps surae

Fig. 4 illustrates the typical behaviour of an FF (fast contracting, fast fatigue) unit. AHPs of the FF units were generally of durations of the same magnitude as those of the FR units. The two unit types were also quite similar with respect to twitch contraction (Table 1), although the fastest twitches encountered in the present study (contraction time = 11 ms) were found among the FF units. The mean values for HRT on the other hand were found to be significantly different ( $p < 0.001$ ) for the FF and FR units (Table 1).

During the "fatigue test" (Fig. 4 D) a nearly complete extinction of FF unit response after 2 min of stimulation was not unusual, but any fatigue index within the limits of 0.25 for FF units, i.e.  $< 0.25$  might be encountered. The "fatigue test" resulted either in a steady decline in contractile tension or more often, in an initial decrease in tension temporarily interrupted by an increase before the final prevalence of the fatigue (Fig. 4 D). The effect was possibly due to the interaction of two physiological phenomena evoked by repetitive stimulation, namely fatigue and potentiation (cf. von Euler and Swank 1940). A "pause test" usually allowed the units to retain some tension for 5–10 s (Fig. 4 D) before the absolute values of contractile tension being quite small however both before and after the test. Those FF units in which some contractile tension still remained 30 s after the "pause test" were immediately exhausted when the "tetanus test" was commenced (Fig. 4 D) no case did the t/p tension ratio exceed 1.00.

#### "Unclassified" units of triceps surae

Sixteen of the 105 units studied in triceps surae had "fatigue indices" between 0.25 and 0.5 and were therefore regarded as "unclassified" units (Burke *et al.* 1973). All of these units had fast twitches and the AHP durations were not significantly different from those of the FF and FR units (Fig. 8 Table 1). The mean value for HRT however was larger than that



These units were submitted to the same series of tests as the extensor motor units, the patterns obtained were quite different in certain respects. So far then, an identity can be established between the motor unit types of triceps surae and the pretibial flexors, and studies are needed to investigate this problem (cf Mosher *et al.* 1972). Similar results were also obtained in studies of kitten motor units (Hammarberg and Kellerth 1972).

#### *Afterhyperpolarization (AHP)*

The present study confirms the observation that motoneurons supplying the slow twitch muscles are generally characterized by more long-lasting AHP's than motoneurons in muscles (Eccles *et al.* 1958, Kuno 1959). However the S units of the pale muscles had AHP's of long duration, although not as long as those of the SOL units. No significant difference in AHP duration was found to exist between FF FR and "unclassified" units. The absolute values for AHP's were found to be considerably greater than those reported by several previous investigators (Kuno 1959, Burke 1967, Prosser and Walke 1974), were generally consistent with those reported by Eccles *et al.* (1958). A possible explanation for this may be that Eccles *et al.* (1958), like ourselves, measured AHP duration to the onset of the terminal after-depolarization which follows the AHP (Lloyd 1951), while other investigators measured the AHP to the point where it passed from hyperpolarization to the initial level. One would expect the contribution of recurrent Renshaw-inhibition to AHP to be negligible in the present study since, first, this inhibition is usually of shorter duration than the AHP (Renshaw 1946) and, second, action potentials were elicited by intracellular stimulation of single neurons rather than by massive antidromic activation of whole motoneuron pools.

#### *Contraction time*

Significant differences in mean values for both contraction time and HRT were observed between gastrocnemius FF FR "unclassified" units, the gastrocnemius S units and the soleus units. Furthermore, the HRT mean values were also significantly different between the FF and FR types of units. In no case did the contraction time of a fast twitch unit exceed 50 ms, and in no case was the corresponding value for an S unit less than 40 ms. Burke (1967) also observed that no motor units in triceps surae had contraction times between 30 and 40 ms and Prosser and Walke (1974) found that the contraction time separating the FF (FF and FR) and slow (S) units was about 40 ms. In general, the contraction times observed in the present study were considerably shorter than those reported by Burke *et al.* (1973, 1974), and somewhat shorter than those reported by Prosser and Walke (1974), but were quite consistent with the values reported by Burke (1967) and Baguet (1974). Although differences in muscle temperature might explain in part these differing results (Gordon and Riple 1953, Buller *et al.* 1968), it should also be pointed out that in the study by Burke *et al.* (1973) the contraction times were measured on maximally potentiated twitches, which may have affected the results. In fact, Prosser and Walke (1974) found that regardless of motor unit type the contraction time for the majority of units increased by at least 50% after prolonged stimulation.

able increase in contractile tension following the "pause test" and they decreased by  $22.0 \pm 15.0\%$  during the course of the "tetanus test". Such a behaviour would be expected from any of the extensor types of units (Table 1). In addition, the flexor unit had a t/p tension ratio of only 1.00 which was never encountered among the units of the extensors (Table 1).

## Discussion

### *Motor unit types*

In confirmation of the findings of Burke *et al.* (1973, 1974) this study found that by the "fatigue" and "sag tests" the majority of motor units in triceps surae could be divided into the 3 types FF, FR and S. It was also found that each of these motor unit types displayed a typical response to the "tetanus" and "pause tests". A minority of the motor units could not be assigned to any of the 3 major types mentioned above, and were considered "unclassified" (Burke *et al.* 1973). All these "unclassified" units displayed AHP durations and responses to the "pause test" which were quite similar to those of the FF and FR unit types. With respect to their responses to the "fatigue" and "sag tests" however they appeared to form a more or less continuous spectrum between the latter two types, in some cases more closely approaching the FF type, in other cases resembling more closely the type FR units. We are therefore prone to agree with the conclusion of Burke *et al.* (1973) that the fast twitch muscle units of triceps surae are likely to constitute a single population although the graded differences between them appear to be distributed in a bimodal pattern.

It has repeatedly been suggested on both physiological and histochemical grounds that the slow motor units of the soleus may not be equivalent to the type S units found in fast muscles (Nyström 1968 b, Mosher *et al.* 1972, Burke *et al.* 1974, Burke and T. 1974, Hammarberg 1974). The present study found the S units of both muscles to be extremely resistant to fatigue. However, significant differences existed between them: the soleus S units had both longer contraction times, HRT's and AHP durations than those of the gastrocnemius, and the behaviour of gastrocnemius S units during the "pause test" suggested a stronger potentiating effect operative in those units than in the S units of the soleus (cf. Olsson and Swett 1971, Burke *et al.* 1974, Hammarberg and Kellerth 1974). The present findings do not decisively indicate whether these differences between S units of different muscles are qualitative rather than quantitative.

All thirty of the SOL motor units in the study of Burke *et al.* (1974) were classed as type S. In the present investigation about fifty soleus motoneurons were impaled, but in these, all of them innervating slow twitch muscle fibres were lost before all parameters had been recorded. Of the remaining seventeen motor units four were found to display characteristics typical of fast twitch units (Fig. 5 and 8). Fast contracting SOL motor units have been reported earlier (Fig. 4 of Bagust 1974) and may possibly be related to that small number of fibres with atypical histochemical staining properties which have been reported to exist in the cat soleus muscle (see e.g. Ogata 1958, Hammarberg 1974).

Only a limited number of motor units of the flexor muscles were studied in detail.

### Interpretation of results from single motor units and whole muscles

The various tests used here to study single motor units have also been applied to whole muscles in a previous investigation (Hammarberg and Kellherth 1975 a), the relative contribution of various motor unit types to the behaviour of their parent muscles may be compared upon. As would be expected from its homogeneous histochemical composition (Hammarberg 1968 b, Burke *et al.* 1974, Hammarberg 1974), the whole SOL muscle responded in or less the same way as the constituent units. The mean values for contraction time were quite similar for the whole muscles (72 ms) and the single units (75 ms), as were also the mean values for HRT (72 ms and 77 ms, respectively), "fatigue index" (0.89 and 0.94 respectively), t/p tension ratio (1.23 and 1.26, respectively) and "pause effect" (5.6% and 6.1% respectively). In the gastrocnemius and pretibial flexors the mean values for contraction time of the whole muscles were 24 ms and 22 ms respectively while the corresponding mean values for single motor units of the same muscles were 20 ms and 21 ms, respectively. The gastrocnemius muscle had a moderately low mean value for "fatigue index" (0.71), was usually stimulated during the "tetanus test" and showed some recovery during the "pause test" (about 15%). The anterior tibial muscle, on the other hand, displayed greater susceptibility to fatigue (mean value for "fatigue index" 0.49), showed no signs of potentiation during the "tetanus test" and exhibited a marked recovery following the "pause test" (about 29%). These findings are compatible with the histochemical observations of higher FR fibre content and a smaller proportion of FP fibres in the gastrocnemius muscle compared to the tibialis anterior (Hammarberg 1974). As pointed out earlier however one must also consider the possibility that the motor unit types of the flexor and extensor muscles may not exhibit identical responses to the various tests employed.

This study was supported by grants from the Swedish Medical Research Council (project No 2896) and the National Institutes of Health (contract No. 71-01-0-0000-0000).

### References

- BURKE, J. D. 1974, Relationship between motor nerve conduction velocities and motor unit contraction characteristics in slow twitch muscle of the cat. *J. Physiol. (Lond.)* 1974, 252, 269-278.
- BURKE, J. D. M. LEWIS and J. C. LOCK, Post-tetanic effects in motor units of fast and slow twitch muscle of the cat. *J. Physiol. (Lond.)* 1974, 237, 115-121.
- BURKE, J. D., S. KNUTT, D. M. LEWIS, J. C. LOCK and R. A. WINTERMAN, Isometric contractions of motor units in fast twitch muscle of the cat. *J. Physiol. (Lond.)* 1973, 231, 87-104.
- ALLAN, A. J., K. W. RAMATUNGA and J. M. BORTH, The influence of temperature on the contractile characteristics of mammalian fast and slow twitch skeletal muscles. *J. Physiol. (Lond.)* 1964, 196, 82 P.
- ALLAN, R. F., Motor unit types of cat triceps surae muscle. *J. Physiol. (Lond.)* 1967, 195, 141-160.
- ALLAN, R. F. and P. THAMES, The correlation of physiological properties with histochemical characteristics in single muscle units. *Ann. N.Y. Acad. Sci.* 1974, 228, 145-160.
- ALLAN, R. F., D. N. LEVINE, M. BALCHMAN and P. THAMES, Motor units in cat soleus muscle: Physiological, histochemical and morphological characteristics. *J. Physiol. (Lond.)* 1974, 238, 503-514.
- ALLAN, R. F., D. N. LEVINE, P. THAMES and P. E. ZAJAC, III, Physiological types and histochemical profiles in motor units of the cat gastrocnemius. *J. Physiol. (Lond.)* 1973, 234, 723-748.
- ALLAN, R. F., D. N. LEVINE, P. E. ZAJAC, III, P. THAMES and W. K. ENOKI, Mammalian motor units: Physiological-histochemical correlations in three types of cat muscle.

### Fatigue properties

The change in contractile tension which occurs in a muscle or motor unit as a result of repetitive stimulation may not depend solely upon the fatigue properties of the unit, but also upon potentiation (von Euler and Swank 1940). This has important implications for the interpretation of fatigue indices. Burke *et al.* (1973) found both the FF and FR units to be potentiated by repetitive stimulation. Olson and Swett (1971) on the other hand, found a considerable difference between their two types of fast contracting units, the one of which probably corresponded to the FF type showing little or no potentiation.

In the present investigation the "tetanus test" caused a visible potentiation of the FF type unit. In the latter case, however, an existing potentiation may have been concealed by the heavy fatigue component since signs of potentiation were usually seen in the early phase of the "fatigue test" (Fig. 4). The motor units of the SOL muscle are known to be little potentiated in comparison with the fast contracting units (Standaert 1964, Åström 1968 a, Olson and Swett 1971, Burke *et al.* 1974). The "fatigue test" would therefore be expected to reflect the pure fatigue component more accurately in the SOL motor units than in the other motor units. This fact should be kept in mind when comparing the fatigue indices of different motor unit types.

The t/p tension ratio was found to differ considerably between fast twitch and slow twitch muscle units, and to a smaller extent also between the type S units of the soleus and gastrocnemius muscles (Fig. 7, Table 1). The former difference may simply reflect the differences in contraction time between fast and slow motor units. With the stimulation frequency used the duration of each pulse train during the pre-tetanic stimulation would be sufficient to allow the fast twitch units to reach their maximal tension. The S units, on the other hand, need longer tetanic stimulation in order to build up their peak tension (Åström and Gersten 1967), which will here be close to tetanic fusion (Mosher *et al.* 1972), and will therefore display greater t/p tension ratios. The observation that, despite their slow contraction times, the S units of the gastrocnemius muscle displayed a somewhat lower mean value for t/p tension ratio than did the SOL units ( $p < 0.05$ , Fig. 7, Table 1), may be explained by the existence of potentiation in the former group of units only (Burke *et al.* 1974).

The difference between the S and FR types of units with respect to recovery after the "pause test" is apparently not related to the state of fatigue only (Table 1). Thus, when the contractions of a type S unit decreased in amplitude during the "fatigue test" there was only negligible recovery after the pause. On the other hand, the FR units always showed an increase in tension after the pause, even in the cases where the fatigue index was close to 1.00. One explanation for this may be a greater ability of the FR units to recover from fatigue, thereby allowing a previously concealed potentiation to manifest itself. It may be significant, in this connection, that the intracellular enzymes of the FR muscle tend toward peripheral concentration, i.e. close proximity to the extracellular capillary supplies, whereas there is a more homogeneous intracellular distribution of enzymes in type S muscle fibres (Olson and Swett 1966, Burke *et al.* 1973, Hammarberg 1974). A more important, however, may be a relatively greater capacity of the FR fibres to regenerate their ATP supplies due to their larger contents of creatine phosphokinase (V. R. Edgerton, personal communication).

## The Postnatal Development of Some Twitch and Fatigue Properties of Single Motor Units in the Ankle Muscles of the Kitten

By

C. HAMMARBERG and J-O KELLERTH

Received 11 March 1975

---

### Abstract

---

AMBERG, C. and J-O KELLERTH. *The postnatal development of some twitch and fatigue properties of single motor units in the ankle muscles of the kitten.* Acta physiol. scand. 1975, 95, 243-257.

Electrical stimulation of single motor units in the gastrocnemius, soleus and pretibial flexor muscles were elicited in kittens bearing 1, 2, 6 and 10 weeks of age. The aim was to establish the pattern of postnatal differentiation of the adult motor unit types (cf. *et al.* 1973, 1974, Hammarberg and Kellerth 1975 a). In the developing type S units of the soleus the mean value for contraction time showed a transient decrease during the early postnatal period, the twitch half-relaxation time (HRT), the duration of motoneurone post-spike afterhyperpolarization and the susceptibility to fatigue remained virtually unchanged during the age period studied. In units of the developing fast twitch muscles the contraction time, HRT and susceptibility to fatigue were already different from those of the soleus units already at 1 week of age. At 6-10 weeks of age the adult characteristics typical of the adult type FF and FR units were attained. The AHP durationally decreased up to 10 weeks of age, and it was then considerably shorter than both at 1 week of age and in the adult stage.

---

Histochemical observations suggest that fibres of the kitten hind limb muscles display differences in fibre size and enzyme content even before 1 week of age (Karpati and Engel 1971, Nyström 1968 c, Hammarberg 1974). Although these muscles have been reported to exhibit certain similar contractile properties during the first postnatal week (Baron 1922, Gray-Brown 1929, Buller *et al.* 1960 a, Buller and Lewis 1965), functional differences between them have also been described, particularly in their contractile responses to repetitive stimulation (Buller and Lewis 1965, Nyström 1968 a, Hammarberg and Kellerth 1975 a). This latter difference is of special interest, since the response patterns of single motor units to various modes of repetitive stimulation have been found to allow a functional classification of different motor unit types in the adult cat (Olsson and Swett 1971, Burke *et al.* 1971, 1973, 1974, Hammarberg and Kellerth 1975 b). The extent to which histochemical and functional dissimilarities between immature kitten muscles may reflect an incipient differentiation into various motor unit types, however, could be determined only by studies at the single unit level.

- DEVANANDAM, M. S., R. M. ECCLES and R. A. WESTERMAN Single motor units of mammalian *J. Physiol. (Lond.)* 1965 178 359-367
- ECCLES, J. C., R. M. ECCLES and A. LUNDBERG The action potentials of the alpha motoneurons in fast and slow muscles. *J. Physiol. (Lond.)* 1958. 142 275-291
- EULER, U. S. v and R. L. SWANK, Tension changes during tetanus in mammalian and non-mammalian. *Acta physiol. scand.* 1930. 1 201-III
- GORDON, G. and C. G. PHILLIPS, Slow and rapid components in a flexor muscle. *Quart. J. exp. Biol.* 1953. 38. 35-45
- GOSLOW Jr G. E., E. K. STAUFER, W. C. NEMETH and D. G. STUART Diga flexor muscle in cat. Their action and motor units. *J. Morph.* 1972. 137 335-352.
- HAMMARBERG, C., Histochemical staining patterns of muscle fibres in the gastrocnemius, semitendinosus and anterior tibial muscles of the adult cat, as viewed in serial sections stained for lipids and succinate dehydrogenase. *Acta Neurol. Scandinavica.* 1974 50, 272-284.
- HAMMARBERG, C. and J.-O. KELLERTH, The postnatal development of some twitch and fatigue properties of the ankle flexor and extensor muscles of the cat. *Acta physiol. scand.* 1975 85 166-178.
- HAMMARBERG, C. and J.-O. KELLERTH The postnatal development of some twitch and fatigue properties of single motor units in the ankle muscles of the kitten. *Acta physiol. scand.* 1975 b. 85 243-251.
- HENNEMAN, E. and C. B. OLSON, Relations between structure and function in the design of skeletal muscle. *J. Neurophysiol.* 1965. 28 581-598
- KUNO, M., Excitability following antidromic activation in spinal motoneurons supplying cat muscles. *J. Physiol. (Lond.)* 1959 149 374-393
- LYDD, D. P. C., After-currents, after potentials, excitability and ventral root electrotonic spread in motoneurons. *J. gen. Physiol.* 1951 35 289-321
- MCPHEDRAN, A. M. R. B. WURZELER and E. HENNEMAN, Properties of motor units in a heterogeneous muscle (soleus) of the cat. *J. Neurophysiol.* 1965. 28 71-84.
- MOSELEY, C. G. R. L. GIERACH and D. G. STUART Soleus and anterior tibial motor units in the cat. *Brain Res.* 1972. 44 1-11
- NYSTRÖM, B., Effect of direct tetanization on twitch tension in developing cat leg muscles. *Acta Neurol. scand.* 1968 a. 74 319-330.
- NYSTRÖM, B., Histochemistry of developing cat muscles. *Acta Neurol. Scandinavica.* 1968 b. 44 403-414
- OGATA, T. A histochemical study of the red and white muscle fibers. *Acta Med. Okazaki.* 1958. 12. 216-227
- OLSON, C. B. and C. P. SWETT Jr A functional and histochemical characterization of motor units in a heterogeneous muscle (flexor digitorum longus) of the cat. *J. comp. Neurol.* 1966. 126. 475-491.
- OLSON, C. B. and C. P. SWETT Jr Effect of prior activity on properties of different types of motor units. *J. Neurophysiol.* 1971 34 1-16.
- PACIOREK, U. and P. M. E. WATZ, Properties of types of motor units in the medial gastrocnemius muscle of the cat. *Brain Res.* 1974 67 89-101
- REINSHAW, B., Central effects of centripetal impulses in axons of spinal ventral roots. *J. Neurophysiol.* 1946. 9 191-204
- SIXTON, A. W. and J. W. GERSTEN, Isometric tension differences in fibers of red and white muscles. *J. Neurophysiol.* 1967 357 199
- STANDERT F. G. The mechanisms of post tetanic potentiation in cat soleus and gastrocnemius muscles. *J. gen. Physiol.* 1964 47 987-1001
- STEPHENS, J. A. and D. G. STUART The classification of motor units in cat medial gastrocnemius muscle. *J. Physiol. (Lond.)* 1974. 240. 43 P
- WURZELER, B., A. M. MCPHEDRAN and E. HENNEMAN Properties of motor units in a heterogeneous muscle (m. gastrocnemius) of the cat. *J. Neurophysiol.* 1965 28. 85-99

ed. It is seen that a considerable variation in duration of AHP was already present at birth of age, the values ranging between 60 and 180 ms. However no significant difference was found between motoneurons innervating the potentially fast twitch and slow twitch muscles, the mean values for AHP duration being  $122.3 \pm 31.9$  ms and  $125.7 \pm 21.4$  ms respectively.

The AHP of the SOL motoneurons changed its mean duration very little as kitten age increased, although there were too few observations in the older age groups to allow any definite conclusions. In the adult animals, however the soleus AHP's were of significantly longer duration ( $198.0 \pm 30.0$  ms) than in the youngest kittens ( $p < 0.001$ ). The mean values for AHP duration of motoneurons innervating the potentially fast twitch muscles remained nearly unchanged during the first 2 postnatal weeks ( $125.2 \pm 16.7$  ms at 2 weeks of age). In the older kittens, however a gradual and conspicuous decrease in duration of the AHP's took place, the lowest values being encountered at around 10 weeks of age ( $61.2 \pm 14.0$  ms). In the adult animals, however the mean duration of AHP of the fast twitch FF + FR units increased significantly ( $105.5 \pm 34.3$  ms,  $p < 0.001$ ). Although the adult values for AHP duration seemed to be attained at a rather late postnatal stage for each motoneuron type, the differences in mean value for AHP duration between the "tonic" and "phasic" motoneurons was rather well established by 10 weeks of age, the mean values here being  $132.4 \pm 36$  ms and  $61.2 \pm 14.0$  ms ( $p < 0.001$ ), respectively.

#### Contraction time (time-to-peak)

Changes in the speed of the muscle unit contractions with increasing age have been illustrated in Figs. 1 and 6. In the youngest kittens the values for contraction time varied between 30 and 80 ms, and even at this age the SOL units were clearly situated on the slow twitch side of the spectrum (Fig. 1). In fact, the mean values for contraction time of the SOL units were quite similar in the 1 week-old kittens ( $75.1 \pm 7.6$  ms) and adult cats ( $76.4 \pm 6$  ms). At 2 weeks of age the contraction times had generally decreased ( $56.0 \pm 15.6$  ms,  $p < 0.001$ ). This is consistent with the results of previous studies (Buller *et al.* 1960 a, Mann & Salafsky 1970, Hammarberg and Kellerth 1975 a) where the contraction time of the sole SOL muscle was found to decrease during the first 3 postnatal weeks, and to later increase towards adult values.

The majority of immature muscle units in the potentially fast twitch muscles gradually shortened their contraction times with increasing age. There was a small but significant ( $p < 0.05$ ) difference between the 1-week-old ( $44.8 \pm 8.0$  ms) and 2-week-old ( $36.6 \pm 12.8$  ms) kittens in this respect, and at 6 weeks of age a substantial decrease had occurred ( $27.2 \pm 12.3$  ms,  $p < 0.001$ ). At 10 weeks of age the contraction times displayed a mean value of  $21.8 \pm 4.7$  ms, which is similar to that of the fast twitch muscle units of adult cats (Hammarberg and Kellerth 1975 b). In some units of these muscles the slow contractions persisted throughout the postnatal period. The fatigue properties of a number of such slow twitch units were investigated at 6 and 10 weeks of age, and all of these units were found to exhibit a behaviour more or less identical with that of the adult type 3 units (Hammarberg and Kellerth 1975 b). Fig. 1 also shows the relationship between motoneurons AHP and twitch time-to-peak

The aims of the present investigation are to describe the pattern of postnatal differentiation of motor units into the various adult unit types (cf Burke *et al.* 1973, 1974; Hammarberg and Kellerth 1975 b) and to compare the behaviour of single units to that of the responding whole muscles (Hammarberg and Kellerth 1975 a). The results will be related to subsequent ultrastructural studies of the postnatal changes in synaptic relations of different types of motoneurons (Ronnevi and Conradi 1974, Hammarberg and Kellerth and Ronnevi in preparation).

### Methods

The general methods used in the present experiments have been described in detail in previous papers (Hammarberg and Kellerth 1975 a, b). Briefly kittens on pentobarbitone anaesthesia (35–40 mg/kg) were ventilated artificially and held in a frame immobilizing the vertebral column and the right hind limb. The medial gastrocnemius (MG), lateral gastrocnemius (LG), soleus (SOL), anterior tibial (TA), and digitorum longus (EDL) and peroneus longus (LP) muscles were dissected free and their tendons arranged for separate isometric connection to a strain gauge myograph. The MG, LG + SOL, and EDL + LP nerves were prepared in the popliteal fossa and mounted intact on stimulating double tungsten electrodes. A lumbo-sacral laminectomy was performed and the dorsal roots were reflected medially to allow a direct approach to the ventral grey matter. In the older kittens the dorsal roots L5–S2 on the right side were cut but in animals less than 3 weeks of age the roots were kept intact in order to permit orthodromic stimulation of the motor pools, since antidromic in action of the motoneurons is often not possible at that age (Kellerth *et al.* 1971). Exposed tissues were covered with mineral oil. Both body temperature and temperature of the oil pools were maintained by infrared light and monitored continuously by thermocouples because of small oil volumes and small body sizes, and variations between 34 and 37°C in the kittens. Temperature control was often difficult in the younger kittens because of small oil volumes and small body sizes, and variations between 34 and 37°C in the accepted. The measured value of contraction time of each muscle unit (= muscle fibres innervated by a single motoneuron) was later adjusted to the corresponding value at 38°C by using the correction factor  $T_{38} = 1.55$  (Gordon and Phillips 1953; Bulter *et al.* 1968). Intracellular micropipette electrodes were used to record from and to stimulate single motoneurons innervating the dissected muscles. In the younger age groups only a minor proportion of the motoneurons would allow an external investigation because of deteriorating. The EMG activity of 21 units, primarily in the 1 and 2 week-old kittens, was recorded using fine monopolar steel wires hooked into the muscle surface over the dimpling produced by the contraction of a unit. Electrical and mechanical responses of the motor units were recorded on an oscilloscope and a DC pen-writer (Devices M2). The testing procedure as well as the stimulation patterns used to evaluate the contractile properties of single muscle units, e.g. the 'T-tube' pause and 'tetanic test' have been described in detail in preceding reports (Hammarberg and Kellerth 1975 a, b).

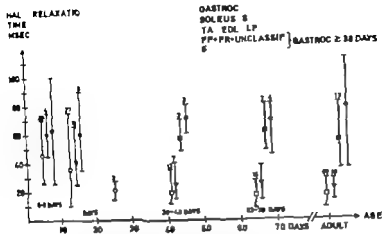
### Results

The present report is based upon a study of 147 motor units in 41 kittens. The selected animals were approximately one week old (mean 7.2 days, S.D. 1.4 days, 17 animals, 17 motor units), two weeks old (14.2  $\pm$  1.1 days, 13 animals, 53 motor units), six weeks old (42.0  $\pm$  3.8 days, 7 animals, 31 motor units) and ten weeks old (67.0  $\pm$  2.4 days, 4 animals, 16 motor units). The motor units investigated belonged to the SOL (26 units), MG (51 units), LG (43 units), TA (2 units), EDL (7 units) and LP (18 units) muscles.

#### *Motoneurone post spike afterhyperpolarization (AHP)*

The durations of motoneurone AHP's which were recorded at various postnatal ages have been plotted along the ordinates in the diagrams of Fig. 1. For comparison, the corresponding values of adult motoneurons (from Hammarberg and Kellerth 1975 b) have also been





2. Graph showing the postnatal changes in twitch half-relaxation time for muscle units of different types. Each bar illustrates the range of observed values, while the superimposed symbols indicate the mean. The number of observations has been indicated above each bar.

#### Twitch half-relaxation time (HRT)

2 shows the values for HRT of muscle units at various postnatal ages. In the animals more than 38 days old the units of Fig. 2 were classified into different types according to criteria discussed below and in a preceding report on adult motor units (Hammarberg & Kellerth 1975 b). In the potentially fast twitch muscle units the mean value for HRT usually decreased with increasing age. In the gastrocnemius units this value changed from  $8 \pm 13.0$  ms at 1 week of age to  $37.3 \pm 16.2$  ms ( $p < 0.1$ ) at 2 weeks of age. At 6 weeks of age the value ( $19.5 \pm 7.8$  ms) had not quite reached that of the 10-week-old kittens ( $16.2 \pm 7.8$  ms) or adult animals ( $15.5 \pm 5.2$  ms,  $p < 0.02$ ). The postnatal changes in HRT of the extensor units followed a time course similar to that of the extensor units, although the mean value was somewhat larger in all the age groups studied.

Only minor changes in HRT mean value occurred in the SOL units during the postnatal period, which observation agrees with previous results from whole SOL muscles (Hammarberg and Kellerth 1975 a). At 1 and 2 weeks of age the mean HRT values of the SOL units were  $62.1 \pm 23.2$  ms and  $60.9 \pm 19.6$  ms, respectively which values do not differ significantly from that of the adults ( $77.3 \pm 26.6$  ms,  $p > 0.1$ ). There was a significant difference in HRT between units of the SOL and gastrocnemius muscles even at 1 week of age ( $p < 0.05$ ). In the older kittens and adult cats the SOL units had a larger mean value for HRT than did the gastrocnemius units classified as being of the S type ( $53.8 \pm 15.3$  ms,  $p < 0.05$  in the adults).

#### Fatigue test

Fig. 3, 4 and 5 show three different motor units which together illustrate the spectrum of motor unit behaviours encountered in the 1-week-old kittens. In A of each figure is seen

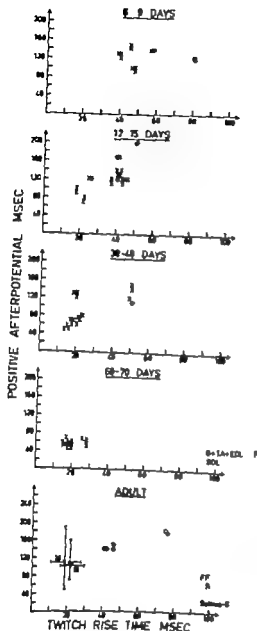


Fig. 1 Graph showing the relationship to motoneurone post spikes after hyperpolarization (rate) and muscle unit contraction time (rise time) for single motor units at different postnatal ages.

at various postnatal ages. In the youngest kittens no relationship at all seemed to exist while at 6 weeks of age a clear change in the direction of the adult situation had occurred. When the regression lines for the values of the three oldest age groups (38-48 days, 60 days and adult animals of Fig. 1) were calculated using the method of least squares, the resulting slopes were 1.66, 1.89 and 1.61 respectively showing that no dramatic change in this relationship occurred after 6 weeks of age. However the corresponding y-intercepts were quite dissimilar in the three age groups (49 ms, 21 ms and 72 ms, respectively) and reflect in particular the general prolongation in motoneurone AHP which occurs after 10th postnatal week (see above).

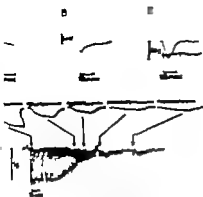


Fig. 4

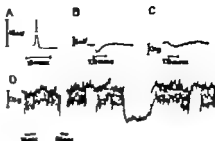


Fig. 5

MG motor unit of a 6-day-old kitten. *A*, Motoneuron action potential. *B*, Motoneuron AHP. *C*, Continuous EMG of the contractile tension elicited by intracellular single-pulse stimulation of the motoneurons. *D*, Continuous EMG of the contractile tension produced by the muscle unit during the "fatigue", "pause" and "tetanus" (bottom), and simultaneously at faster time base, the EMG records and tensions produced during the test sequence at the moments indicated by arrows (top).

SOL motor unit of an 8-day-old kitten. *A*, Motoneuron action potential. *B*, Motoneuron AHP. *C*, Continuous EMG of the contractile tension elicited by intracellular single-pulse stimulation of the motoneurons. *D*, Continuous EMG of the contractile tension produced by the muscle unit during the "fatigue", "pause" and "tetanus" (bottom), and simultaneously at faster time base, the EMG records and tensions produced during the test sequence at the moments indicated by arrows (top).

#### the test

At 2 min of the "fatigue test" had elapsed each unit was allowed to rest for 10 s, after which the iterative stimulation was resumed (see Fig. 3, 4 and 5). Changes in contractile

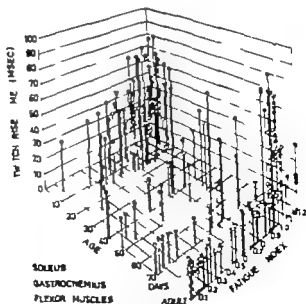


Fig. 6. Three-dimensional plot showing the relationship between contraction time (vertical axis), "fatigue index" (horizontal Z axis) and postnatal age (horizontal X-axis) for muscle units of different muscles.

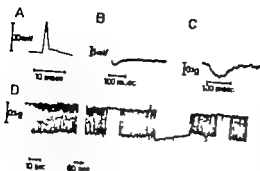


Fig. 3 MG motor unit of a 6-day-old kitten. *A*, Motoneurone action potential. *B*, Motoneurone afterhyperpolarization. *C*, Twitch contraction elicited by intracellular stimulation of the motoneurone. *D*, Continuous recording of the contractile tension produced by the unit during the fatigue "pause" and "tetanus" tests.

the motoneurone action potential, in *B* the motoneurone AHP, in *C* the twitch contraction and in *D* the contractile responses of the muscle unit to the "pause" and "tetanus tests" (Hammarberg and Kellerth 1975 a, b). Despite the differences in some of their responses, particularly in those illustrated in *D* of Fig. 3, 4 and 5. For example, the fast twitch (contraction time = 32 ms) MG unit of Fig. 4 was extremely susceptible to fatigue (Fig. 4 *D*), while the slow twitch (contraction time = 89 ms) SOL unit of Fig. 5 was quite fatigue resistant (Fig. 5 *D*). The unit of Fig. 4 however represents a rather extreme case of fatigue sensitivity at this age, while a more "normal" behaviour at this age is exhibited by the MG unit shown in Fig. 3.

The "fatigue indices" (Burke *et al.* 1971, 1973; Hammarberg and Kellerth 1975 a, b) on the whole rather large in the smallest kittens, indicating a well developed resistance to fatigue in the majority of immature units (Fig. 6). Of the 69 units studied in kittens at 1 and 2 weeks of age, 52 had fatigue indices exceeding 0.75 (Fig. 3, 5 and 6). In the cat this value has been used as the lower limit for classifying the fatigue resistant FM units (Burke *et al.* 1971, 1973; Proske and Walke 1974; Hammarberg and Kellerth 1975 b). However, even in the smallest kittens some units were quite susceptible to fatigue (Fig. 4 and 6) which indicates the possibility that the differentiation into adult motor unit types had already commenced. It should also be pointed out that even in the 1 and 2-week-old kittens the SOL units were among the most fatigue resistant, with a mean value of "fatigue index" of  $1.00 \pm 0.11$ . This value is larger than that of the gastrocnemius units ( $0.73 \pm 0.21$ ,  $p < 0.001$ ) at the same age. Compared with the gastrocnemius units the corresponding value for the flexor units of the youngest kittens was surprisingly large ( $0.91 \pm 0.12$ ,  $p < 0.001$ ), but one should realize that in certain other respects also (see below, Hammarberg and Kellerth 1975 b) the flexor unit types may not be quite equivalent to the extensor units. With increasing age a gradual differentiation took place among the units of the fast twitch muscles. This development is reflected in the larger proportion of fatigue-sensitive units ("fatigue index"  $< 0.25$ ) found at 6 and 10 weeks of age (Fig. 6). The units in fatigability with increasing age, showed only non-significant ( $p > 0.1$ ) changes in the mean value for "fatigue index" being  $0.97 \pm 0.06$  at 6–10 weeks of age, and  $0.94 \pm 0.06$  in the adult animals.



Fig. 4

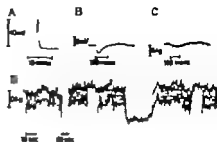
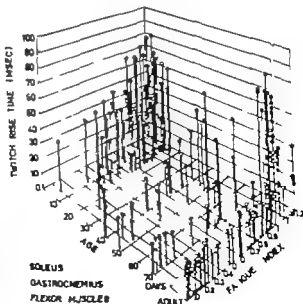


Fig. 5

1 MG motor test of 6-day-old lizards. *A*, Motor unit action potential. *B*, Motor unit AHP  
 2 and contraction elicited by intracellular single-pulse stimulation of the motor unit. *C*, Continuous  
 3 recording of the contractile tension produced by the muscle test during the "fatigue" "pause" and "release"  
 4 (bottom), and simultaneously at faster time base, the EMG records and tensions produced during  
 5 the test sequence at the moments indicated by arrows (top).

15 SOL motor test of an 8-day-old lizard. *A*, Motor unit action potential. *B*, Motor unit AHP  
 16 and contraction elicited by intracellular single-pulse stimulation of the motor unit. *C*, Continuous  
 17 recording of the contractile tension produced by the muscle test during the "fatigue" "pause" and  
 18 "release" (bottom), and simultaneously at faster time base, the EMG records and tensions produced during  
 19 the test sequence at the moments indicated by arrows (top).

21 2 min of the "fatigue test" had elapsed each unit was allowed to rest for 10 s, after  
 22 which the iterative stimulation was resumed (see Fig. 3, 4 and 5). Changes in contractile



23 6. Three-dimensional plot  
 24 showing the relationship between  
 25 contraction time (vertical  
 26 Z-axis), "fatigue index" (hor-  
 27 izontal X-axis) and postural an-  
 28 gles (vertical Y-axis) for muscle  
 29 groups of different muscles.

tension immediately following this pause were studied. In the adult animals usually allowed fast twitch units to increase their contractile tension, and then to differentiate between FR and S units (Hammarberg and Kellerth 1975 b).

The "pause effect" measured in single units have been plotted along the abscissa of Fig. 7. With one exception, the values ranged between 0-20% in the age group (6-9 days), but one week later (12-15 days) they have started to spread, a tendency that is even more pronounced at 6-10 weeks of age. In the diagrams of Fig. 7 With one exception, the values ranged between 0-20% in the age group (6-9 days), but one week later (12-15 days) they have started to spread, a tendency that is even more pronounced at 6-10 weeks of age. In the units at 38-70 days of age and in the adult stage have been omitted, since, due to tensions usually produced by these units by the time of the "pause test" (see Hammarberg and Kellerth 1975 b) considerable error was often introduced into the of the relative changes in contractile strength.

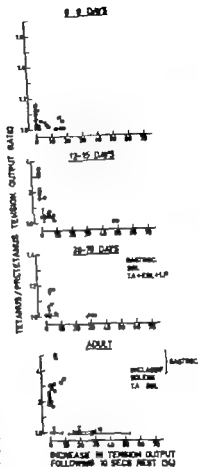
It is evident from Fig. 7 that the fatigue resistant type S units were grouped part of the spectrum at all ages, which means that they remained more or less unaffected by the "pause test" throughout the postnatal period. The fast twitch extensor units, on the other hand, became more affected by the pause with increasing age. In the adult amount of recovery in contractile tension following the 10 s pause was not significantly ( $p > 0.1$ ) related to the state of fatigue of the units. This is also illustrated in Fig. 7. FR units ("fatigue index"  $> 0.75$ ) and "unclassified" units ("fatigue index"  $< 0.25$ ) of the extensor muscles showed a considerable overlap in "pause effects" (see also Hammarberg and Kellerth 1975 b).

The units of the flexor muscle responded to the "pause test" in a way quite unlike that of the extensor type S units, i.e. the effects were small in all age groups studied. This was quite unexpected considering both the rather wide range of "fatigue indices" by the flexor units (Fig. 6) and the large "pause effect" obtained in whole flexor (Hammarberg and Kellerth 1975 a). However the flexor units of Fig. 7 probably represent a skewed sample since as many as thirteen of the fifteen units in the 38-70-day-old and adult cats were identified as FR or "unclassified" units with "fatigue indices" between 0.62 and 0.97. It may be concluded from Fig. 7 though, that these units respond to the "pause test" quite differently than did the corresponding motor unit type S extensor muscles. The large "pause effect" found in the study of whole flexor (Hammarberg and Kellerth 1975 a) may possibly be attributed to FF units which are represented in Fig. 7.

#### "Tetanus test"

The "fatigue indices" in kittens around 1-2 weeks of age indicated that the muscle units of the gastrocnemius were generally more sensitive to fatigue than those of the SOL and rectus abdominis muscles (see above). A similar tendency was also found with the tetanus test, where the relative decrease in tension ( $39.6 \pm 21.7\%$ ,  $n = 15$ ) during the course of sustained stimulation, than did the units of the pretibial flexors ( $24.3 \pm 20.2\%$ ,  $n = 13$ ,  $p < 0.1$ ). However there was no significant difference in the decrease in tetanic tension between the units of the gastrocnemius and SOL ( $29.4 \pm 14.2\%$ ,  $n = 12$ ,  $p > 0.1$ ) muscles of this age.

At 6-10 weeks of age the tension of the SOL units decreased by only  $4.5 \pm 2.1\%$  ( $n = 10$ )



Graphs showing the posttetanic changes in the relation between tetanus/pretetanic tension ratio (ordinate) and increase in tension output following 10 sec rest (abscissa) of muscle units of different muscles.

In the adult animals the values for tetanic tension decreases were  $0.4 \pm 1.2\%$  ( $n=8$ ) for the SOL units and  $89.7 \pm 9.9\%$  ( $n=10$ ) for the gastrocnemius "unclassified" units (Hammarberg and Kellerth 1975 b). In contrast to the situation in the kittens, both the FR and SOL units of the adult gastrocnemius showed an average decrease in tension during the tetanus of  $24.4 \pm 29.1\%$  ( $n=13$ ) and  $7.4 \pm 10.2\%$  ( $n=7$ ), respectively due probably to potential effects induced by the tetanic stimulation (Standert 1964, Nyström 1968 a, Ohlsson 1971, Hammarberg and Kellerth 1975 b). The FR units of the flexor muscles, on

other hand, decreased in tetanic tension in all the cases studied ( $22.0 \pm 15.1$  Hammarberg and Kellerth 1975 b).

In adult animals, as seen in Fig. 7 the tetanus/pretetanus (t/p) tension: "pause test" clearly separated the gastrocnemius FR and S types of units (Hammarberg and Kellerth 1975 b). In the 1 week-old kittens such a separation was not possible only one week later a clear tendency for arrangement according to the adult pattern was seen (Fig. 7). In the older kittens, 6 and 10 weeks of age, the responses were quite similar to those of mature muscle units. The gastrocnemius S units in the adult cats used somewhat larger t/p tension ratios ( $1.35 \pm 0.12$ ) than did the SOL units ( $1.26 \pm 0.10$ ) due possibly to the existence of potentiation in the former unit type only (Burke and Fessenden 1974). A similar difference can also be seen in the oldest kittens (Fig. 7) where the corresponding ratios for gastrocnemius and SOL type S units were  $1.37 \pm 0.04$  and  $1.26 \pm 0.01$  respectively. The behaviour of the flexor units was quite different from that of the extensor units: neither the "pause test" nor the "tetanus test" significantly affected the tension output of the flexor units, which, often were also surprisingly resistant to fatigue in the older kittens and adult cats (see above).

#### *EMG activity during fatigue*

The electromyographic activity of 21 units of the triceps surae muscle was recorded. The majority of these units were from kittens belonging to the two youngest age groups. The purpose of the EMG recordings was to ascertain whether or not the fatigue of single units was accompanied by a change in EMG response which would indicate a failure of factors other than the contractile mechanism might have been responsible for the decrease in contractile tension. In all units where fatigue was present there was, with the exception, a concomitant decrease in amplitude of the EMG potentials. The only unit which exhibited an increase in EMG amplitude during the fatigue. Generally the EMG amplitude in contractile tension of a muscle unit was larger relatively ( $p < 0.02$ ) than the EMG amplitude (see e.g. Fig. 3). In no case was a "drop out" of spikes observed during the fatigue of EMG potentials, which indicated that the propagation of nervous impulses was adequate down to at least the branching point of the axon terminals.

#### *Discussion*

##### *Motoneurone AHP*

The AHP's of motoneurons innervating potentially fast twitch muscle fibres were found to gradually decrease in duration with increasing age. This decrease may to some extent be attributed to postnatal changes in the permeability properties of the motoneurone membrane, such as e.g. an increased efficiency of the ionic pumps. Similarly motoneurone resting membrane potentials and action potentials have been found to increase in magnitude with age and to display adult values around 6 weeks postnatally (Kellerth *et al.* 1974). However the apparent absence of postnatal changes in soleus AHP's is more difficult to reconcile with permeability change, unless one assumes differences in the AHP generating mechanisms and/or in the state of maturity at birth between the two groups of motoneurons.



general increase in AHP duration which occurred in all motoneurons after 10 weeks was quite conspicuous, but one can only speculate about the mechanism underlying this age more or less mature conditions would be expected with respect to motor membrane properties (Kellerth *et al.* 1971), extracellular concentration of electrolytes (Thelen 1973) and recurrent inhibitory effects (Mellström 1971). However although the cell body volume of lumbar motoneurons increases relatively little after 5-6 weeks of age (Mellström and Skoglund 1969), the branching of their dendritic trees has been reported to continue until 4-5 months of age (Scheibel and Scheibel 1970). Such an increase in distal dendritic membrane area may account for the prolongation of motor AHPs in the older kittens.

#### Contraction

Age-related changes in twitch contraction time and HRT were generally similar to those obtained in a previous study on whole muscles (Hammarberg and Kellerth 1975 a). In addition the mean ages for contraction time corresponded closely to those observed in the SOL and flexor digitorum longus muscles at 2 and 6 weeks of age by Bagust (1974). The values for twitch speed and HRT were quite scattered even in the smallest kittens, possibly reflecting histochemical variations among muscle fibres at these ages (Hammarberg 1974), but the wide range of values typical of the adult animal was not established until around 6 weeks of age, at which time most fast units displayed adult twitch characteristics (cf Bagust *et al.* 1974). At this same age the difference in twitch duration between type FR and type S units was also close to that of the adult units (see Fig. 1 and 2). Considering the fact that contraction characteristics are at least partly dependent upon the type of neurone innervating the muscle fibres (Buller *et al.* 1960 a, b) one should expect that polynervous innervation (Bagust *et al.* 1973) as well as formation of new axonal sprouts (Bagust *et al.* 1974) have been reported to occur in the kitten hind limb muscles until 6 weeks of age. Only around this age, therefore, would all muscle fibres be exposed to a constant and fully developed neuronal influence which would allow them to achieve final adult characteristics.

#### Motor properties

At 1-2 weeks of age considerable variations were encountered in the responses of motor units of the different muscles to the "fatigue", "pause" and "tetanic" tests. This may be explained by the fact that differentiation between various motor unit types had already commenced, although response patterns entirely typical of the adult FF, FR and S units (Hammarberg and Kellerth 1975 b) were not in the majority until 6-10 weeks of age. A relatively large proportion of "unclassified" units was in evidence even at this age, however, suggesting that maturation was not yet complete. The SOL units were generally found to be among the least fatiguable even at 1-2 weeks of age, but the presence of potentiation in the L muscles of the smallest kittens (Nystrom 1968) may have concealed a "true" muscular fatigue (cf Hammarberg and Kellerth 1975 a). With the stimulation frequencies used here, post-tetanic potentiation of muscle contractions in both kittens and adult cats (Nystrom 1968 a) as well as fatigue of mature muscle

other hand, decreased in tetanic tension in all the cases studied ( $22.0 \pm 15.0$ , Hammarberg and Kellerth 1975 b).

In adult animals, as seen in Fig. 7 the tetanus/pretetanus (t/p) tension ratio "pause test" clearly separated the gastrocnemius FR and S types of units (Hammarberg and Kellerth 1975 b). In the 1 week-old kittens such a separation was not possible, only one week later a clear tendency for arrangement according to the adult pattern seen (Fig. 7). In the older kittens, 6 and 10 weeks of age, the responses were quite like those of mature muscle units. The gastrocnemius S units in the adult cats usually had somewhat larger t/p tension ratios ( $1.35 \pm 0.12$ ) than did the SOL units ( $1.26 \pm 0.06$ ), due possibly to the existence of potentiation in the former unit type only (Borke *et al.* 1974). A similar difference can also be seen in the oldest kittens (Fig. 7) where the corresponding ratios for gastrocnemius and SOL type S units were  $1.32 \pm 0.04$  and  $1.17$  ( $p < 0.01$ ), respectively. The behaviour of the flexor units was quite different from the extensor units: neither the "pause test" nor the "tetanus test" significantly altered the tension output of the flexor units, which often were also surprisingly resistant to fatigue in the older kittens and adult cats (see above).

#### *EMG activity during fatigue*

The electromyographic activity of 21 units of the triceps surae muscle was recorded. The majority of these units were from kittens belonging to the two youngest age groups. The purpose of the EMG recordings was to ascertain whether or not the "fatigue" observed by single units was accompanied by a change in EMG response which would indicate failure of factors other than the contractile mechanism might have been responsible for the decrease in contractile tension. In all units where fatigue was present there was, with exception, a concomitant decrease in amplitude of the EMG potentials. The only unit exhibited an increase in EMG amplitude during the fatigue. Generally the decrease in contractile tension of a muscle unit was larger relatively ( $p < 0.02$ ) than the decrease in EMG amplitude (see e.g. Fig. 3). In no case was a "drop out" of spikes observed, which indicated that the propagation of nervous impulses was adequate down to at least the branching point of the axon terminals.

#### Discussion

##### *Motoneurone AHP*

The AHP's of motoneurons innervating potentially fast twitch muscle fibres were found to gradually decrease in duration with increasing age. This decrease may to some extent be attributed to postnatal changes in the permeability properties of the motoneurone membrane, such as e.g. an increased efficiency of the ionic pumps. Similarly motoneurone resting membrane potentials and action potentials have been found to increase in magnitude with age and to display adult values around 6 weeks postnatally (Kellerth *et al.* 1975). However the apparent absence of postnatal changes in soleus AHP's is more difficult to reconcile with permeability change, unless one assumes differences in the AHP generating mechanisms and/or in the state of maturity at birth between the two groups of motoneurons.

to developmental changes, or alternatively that most developmental changes occur early. With respect to muscle fibre diameters the S type units appear more mature at the potentially fast twitch units (Engel and Karpal 1968, Hammarberg 1974).

### 1. Considerations

When kitten lacks the ability to stand and walk, but develops these functions during the first three postnatal weeks, being able even to run after 5-6 weeks. The postnatal development of the postural and locomotor mechanisms responsible for these functions involves a series of structural and functional changes in both the central and peripheral parts of the nervous system (cf Skoglund 1960 a-e, Berthold 1968, Nystrom 1968 a, b, c, Conradi 1969, Romer and Skoglund 1969, Mellstrom 1971).

The present study found that the muscle units displayed adult functional characteristics from 6 weeks of age. The pattern of changes in histochemical appearance of the muscles follows a similar time course (Hammarberg 1974). This particular age has also been reported by other authors to be a transition point between immature and adult properties in the nervous system, i.e. after 6 weeks of age polynervous innervation of muscle fibres and formation of new axonal contacts does not occur (Bagust *et al.* 1973, 1974), motoneurone membrane potentials and resting membrane potentials show adult magnitudes (Kellerth *et al.* 1971), differences in morphological appearance of the motor nerve terminals of the gastrocnemius and soleus muscle fibres is evident (Nystrom 1968 b) and there is only a relatively small additional increase in motoneurone cell body volume (Mellstrom and Skoglund 1971).

In certain other respects maturity of motor function may occur earlier than 6 weeks. The refractory period of immature fibres is about twice that of mature fibres (Huxley 1939), impulse frequencies can be mediated in a mature way in developing fibres with a 6  $\mu$ m diameter (Ekholm 1967), which in the ventral root fibres corresponds to an age of about 3 weeks (Skoglund and Romer 1965). At this age the ventral root fibres also display adult physiological characteristics in the node-paranode region (Berthold 1968). When considering the role of neurotrophic influences in the differentiation of motor function (Buller *et al.* 1960 a, b), one should also pay attention to the elimination and reformation of synaptic contacts which occur on lumbar motoneurons before 3 weeks of age (Conradi 1968, Rönnevi and Conradi 1974, Conradi and Rönnevi 1975). The possible relation between such changes in synaptic reflex connections and the differentiation of various motor unit types will be considered in subsequent physiological and ultrastructural studies (Hammarberg in preparation, Hammarberg, Kellerth and Rönnevi, in preparation).

This study was supported by grants from the Swedish Medical Research Council (project No 2286) and the Karolinska Institute.

### References

- BAGUST, J., D. M. LEWIS and R. A. WESTERMAN, Polynervous innervation of kitten skeletal muscle. *J. Physiol. (Lond.)* 1971, 229, 241-255.
- BULLER, J., D. M. LEWIS and R. A. WESTERMAN, The properties of motor units in fast and slow twitch muscle during post-natal development in the kitten. *J. Physiol. (Lond.)* 1974, 237, 73-90.

units (Burke *et al.* 1973) appear to be events entirely restricted to the muscle. The fatigue exhibited by muscle units of the 1 and 2 week-old kittens, however, was accompanied by a decreased amplitude of the EMG potentials of the units. For the relative depression in contractile tension was generally much greater than for the EMG responses, a linear relationship between these two factors cannot be taken for granted (Lüttgau 1965) and any direct comparison of the fatigability of immature and adult units is therefore hazardous. There is some support, however, for the view that the differences in fatigue properties between units of the potentially fast and slow twitch of the youngest kittens may not depend on differences in presynaptic mechanisms or in muscular transmission, but rather on postsynaptic differences (see Discussion of Hammarberg and Kellerth 1975 a).

The responses of single units to repetitive stimulation were generally comparable to those of the corresponding whole muscles (Hammarberg and Kellerth 1975 a). An exception, however, was found in kittens around 1 and 2 weeks of age, where single units did not exhibit the same large decrease in tension during the 'tetanus test' as did the whole muscles. This difference between the responses of single units and whole muscles possibly be accounted for by ischemia arising secondarily to an occlusion of the blood supply and/or by extracellular accumulation of metabolites or potassium, during a tetanic contraction of the whole muscles. Whatever the explanation for the above observation may be, it appears that the immature muscles, and particularly the potent twitch ones, are more sensitive to such influence than the adult muscles.

#### *Differentiation of motor unit types*

Statistically significant differences in twitch contraction time, twitch half-relaxation time and susceptibility to fatigue between muscle units of potentially fast and slow twitch were observed at even 1 week of age. This indicates that differentiation into various motor unit types starts at a very early age, possibly even at a prenatal stage (cf. Ridge 1967) though the contractile response patterns typical of the adult FF and FR motor units (Hammarberg and Kellerth 1975 b) are not fully developed until 6-10 weeks of age.

With the exception of contraction time, which showed a transient decrease during the early postnatal period, the functional parameters investigated in the SOL units showed only insignificant changes from 1 week of age to the adult stage. This is consistent with findings in previous studies on whole SOL muscles (Hammarberg and Kellerth 1975) but may seem unexpected in view of the heterogeneity of SOL fibres in the youngest kittens as revealed by histochemical stains for ATPase and lipids (Karpáti and Engel 1967; 1968 c, Hammarberg 1974). Postnatal variation in soleus ATPase activity and its disappearance at 7 weeks of age (Nyström 1968 c) may well account for the simultaneous changes in contraction time, since a direct correlation between these two parameters is known to exist (Bárány 1967). Postnatal changes in lipid content (Hammarberg 1974) may, however, have necessarily been reflected in the types of functional tests used here.

The absence of obvious postnatal changes in functional properties of the type II units contrasts markedly to the pattern of differentiation of the fast twitch units. This indicates that the II units belong to a more primordial unit type which does not undergo

3. B., Postnatal development of motor nerve terminals in "slow-red" and "fast white" cat muscles. *Neural. Scandinavica*. 1968 b. 44. 363-383.
4. B., Histochemistry of developing cat muscles. *Acta Neurol. Scandinavica*. 1968 c. 44. 405-439.
5. C. B. and C. P. SWITT, J. Effect of prior activity on properties of different types of motor units. *Neurophysiol.* 1971. 34. 1-16.
6. U. and P. M. E. WALTZ, Properties of types of motor units in the medial gastrocnemius muscle of cat. *Brain Res.* 1974. 67. 89-101.
7. R. M. A. P. The differentiation of conduction velocities of slow twitch and fast twitch muscle nerve innervations in kittens and cats. *Quart. J. exp. Physiol.* 1967. 52. 293-304.
8. L.-O. and E. CONRAD, Ultrastructural evidence for spontaneous elimination of synaptic terminals from motoneurons in the kitten. *Brain Res.* 1974. 80. 335-339.
9. M. E. and A. B. SCHMIDT, Developmental relationship between spinal motoneuron dendrites and patterned activity in the head limb of cats. *Exp. Neurol.* 1970. 29. 328-335.
10. S. On the postnatal development of postnatal mechanisms as revealed by electromyography in decerebrate cats. *Acta physiol. scand.* 1960 a. 49. 299-317.
11. S., The spinal transmission of proprioceptive reflexes and the postnatal development of conduction velocity in different head limb nerves in the kitten. *Acta physiol. scand.* 1960 b. 49. 318-329.
12. S., The activity of muscle receptors in the kitten. *Acta physiol. scand.* 1960 c. 50. 203-221.
13. S., Central connections and functions of muscle nerves in the kitten. *Acta physiol. scand.* 1960 d. 50. 222-237.
14. S., The reactions to tetanic stimulation of the two-neuron arc in the kitten. *Acta physiol. scand.* 1960 e. 50. 238-253.
15. S. and C. ROSSIGNOL, Postnatal growth of spinal nerves and roots. A morphological study in the kitten with physiological correlations. *Acta physiol. scand.* 1963. 66. Suppl. 262.
16. F. O., The mechanisms of post-tetanic potentiation in cat selen and gastrocnemius muscles. *Am. J. Physiol.* 1964. 47. 987-1001.

- BANU, G. Recherches physiologiques sur le développement neuromusculaire. Paris 1922.
- BÁRÁNY, M. ATPase activity of myosin correlated with speed of muscle shortening. *J. gen. Phys.* 50, 197-218.
- BERTHOLD, C. H., Ultrastructural and light microscopical features of postnatally developing feline peripheral, myelinated nerve fibres. 1968. Brogrenhandt tryckeri AB, Lidsjö.
- BULLER, A. J. and D. M. LEWIS, The rate of tension development in isometric tetanic contractions of mammalian fast and slow skeletal muscle. *J. Physiol. (Lond.)* 1965, 176, 337-514.
- BULLER, A. J., J. C. ECCLES and R. M. ECCLES, Differentiation of fast and slow motoneurons in the limb. *J. Physiol. (Lond.)* 1960 a, 130, 399-416.
- BULLER, A. J., J. C. ECCLES and R. M. ECCLES, Interactions between motoneurons and muscles of the characteristic speeds of their responses. *J. Physiol. (Lond.)* 1960 b, 130, 417-439.
- BULLER, A. J., K. W. RANATUNGA and J. M. SMITH, The influence of temperature on the characteristics of mammalian fast and slow twitch skeletal muscles. *J. Physiol. (Lond.)* 1961, 144, 1-14.
- BURKE, R. E., D. N. LEVINE, F. E. ZAJAC, III, P. TRAVERS and W. K. ENGEL, Mammalian neuromuscular physiological-histochemical correlation in the three types in cat gastrocnemius. *Science* 175, 709-712.
- BURKE, R. E., D. N. LEVINE, P. TRAVERS and F. E. ZAJAC, III, Physiological types and histochemical units in motor units of the cat gastrocnemius. *J. Physiol. (Lond.)* 1973, 234, 723-748.
- BURKE, R. E., D. N. LEVINE, M. SALCMAN and P. TRAVERS, Motor units in cat soleus muscle: Physiological, histochemical and morphological characteristics. *J. Physiol. (Lond.)* 1974, 238, 503-514.
- CONRADI, S., On motoneuron synaptology in cats and kittens. An electron microscopic study of the structure and location of neuronal and glial elements on cat lumbarosacral motoneurons in the normal cat after dorsal root section. 1969. Tryckeribolaget Ivar Håggström, Stockholm.
- CONRADI, S. and L.-O. RÖNNERV, Spontaneous elimination of synapses on cat spinal motoneurons: Do half of the synapses on the cell bodies disappear? *Brain Res.* 1973, 92, 505-518.
- CULLIUM, S., A study of plasma and CSF potassium relationship during postnatal development in the cat. *Acta physiol. scand.* 1973, 89, 394-399.
- DENNY BROWN, H. E., The histological features of striped muscle in relation to its functional properties. *Proc. roy. soc. B* 1929, 104, 371-411.
- ERIKSSON, J., Postnatal changes in cutaneous reflexes and in the discharge pattern of cutaneous and muscle afferents. A morphological and physiological study in the cat. *Acta physiol. scand.* 1967, Suppl. 231.
- ENGEL, W. K. and G. KARPATI, Impaired skeletal muscle maturation following neonatal neonatal muscle denervation. *Biol.* 1968, 17, 713-723.
- GORDON, G. and C. G. PHILLIPS, Slow and rapid components of a flexor muscle. *Quart. J. exp. Physiol.* 1953, 38, 15-43.
- HAMMARBERG, C., The histochemical appearance of developing muscle fibres in the gastrocnemius and anterior tibial muscles of the kitten, as revealed in serial sections stained for lipids and for dehydrogenase. *Acta Neurol. Scandinavica* 1974, 50, 285-301.
- HAMMARBERG, C. and J.-O. KELLERTH, The postnatal development of some twitch and fatigue properties of the ankle flexor and extensor muscles of the cat. *Acta physiol. scand.* 1975 a, 95, 164-178.
- HAMMARBERG, C. and J.-O. KELLERTH, Studies of some twitch and fatigue properties of different unit types in the ankle muscles of the adult cat. *Acta physiol. scand.* 1975 b, 95, 231-242.
- ISURU, J. H., The properties of growing nerve fibers. *Amer. J. Physiol.* 1939, 127, 140-154.
- KARPATI, G. and W. K. ENGEL, Neuronal trophic function. *Arch. Neurol.* 1967, 17, 541-545.
- KELLERTH, J.-O., A. MELLESTRÖM and S. SKOGLUND, Postnatal excitability changes of kitten motoneurons. *Acta physiol. scand.* 1971, 83, 31-41.
- LÖTTIG, U. H. C., The effect of metabolic inhibitors on the fatigue of the action potential in single fibres. *J. Physiol. (Lond.)* 1965, 178, 45-67.
- MANN, W. S. and B. SALATSKY, Enzymic and physiological studies on normal and diseased developing and slow cat muscles. *J. Physiol. (Lond.)* 1970, 208, 31-47.
- MELLESTRÖM, A., Recurrent and antidromic effects on the monosynaptic reflex during postnatal development in the cat. *Acta physiol. scand.* 1971, 82, 490-499.
- MELLESTRÖM, A. and S. SKOGLUND, Quantitative morphological changes in some spinal cord motoneurons during postnatal development. A study in the cat. *Acta physiol. scand.* 1969, Suppl. 231.
- NYSTRÖM, B., Mechanical and electrical responses to single shocks in developing cat leg muscles following tetanization. *Acta physiol. scand.* 1968, 74, 707-725.

1. Secretory responses to gastrin in Pavlov pouch dogs with and without perfusion of the duodenal bulb with 0.1 N HCl.

Number of expts.	Control hours		Mean secretory rate expressed as percentage of the secretory level during the control hour. Half hour periods following the control hour			
	Range of secretory levels, mmol/15 min	Relative S.E. of mean of four 15-min periods (per cent)	1	2	3	4
15						
3	0.45-0.51	9	30	20	39	96
3	0.19-0.67	11	18	8	21	70
3	0.29-0.47	11	67	13	23	101
	Mean $\pm$ S.E. of mean		41 $\pm$ 7	11 $\pm$ 7	28 $\pm$ 6	89 $\pm$ 11

as had been reached, bulbar pouches were perfused with 0.1 M HCl for 1 h. Following completion of the urecholine gastric secretion was followed for another hour. Further details of the procedure of the perfusion have been given elsewhere (Anderson, Nilsson and Uvnäs 1967).

Secretin hindered the bulbar mechanism in each dog, as efficient in inhibiting acid secretion. Control of experiments was performed using porcine gastrin (Anderson and Nilsson 1969) as secretory stimulus. These experiments were carried out in the same way as the experiments with Urecholine. Common methods of analysis of variance were used (Sardeson 1967) for the statistical evaluation of the data.

### Results

Bulbar perfusion with acid profoundly inhibited acid responses to exogenous gastrin in all dogs (Table I). When similar gastric secretory rates were induced by Urecholine in the same dogs, bulbar acidification produced some reduction of the acid output in one dog (A), however the inhibitory pattern was different from that observed in experiments with gastrin where inhibition of acid secretion consistently became most pronounced during the second half-hour of bulbar acidification. In dog B acidification of the bulb did not significantly influence the acid output from the gastric pouches, whereas in dog C an increase in the acid output was observed following perfusion of the bulbar pouch with acid. These results are presented in Table II.

### Discussion

A series of studies in dogs attempts have been made to reveal the characteristics of the mechanism by which acid in the duodenal bulb inhibits acid secretion. From these results it appears that the bulbar mechanism is very effective in inhibiting acid responses to exogenous gastrin (Anderson, Nilsson and Uvnäs 1967; Anderson and Nilsson 1969; Nilsson and Rude 1971; Nilsson 1974; Nilsson 1975 c) or to stimuli causing release of gastrin such as test meals (Anderson and Uvnäs 1961; Anderson and Sjodin 1972; Nilsson 1975 b), or insulin loading (Nilsson 1969; Anderson and Sjodin 1972; Nilsson 1975 a) or insulin hypoglycemia (Nilsson 1969; Anderson and Sjodin 1972). Also in the present study bulbar acidification profoundly inhibited acid responses induced by exogenous gastrin, which shows that the bulbar mechanism was very efficient in inhibiting acid secretion in each

## Effect of Bulbar Acidification on Gastric Acid Responses to Urecholine in Pavlov Pouch Dogs

By

GÖRAN NILSSON

Received 27 March 1975

---

### Abstract

Nilsson, G. *Effect of bulbar acidification on gastric acid responses to Urecholine in pouch dogs.* Acta physiol. scand. 1975. 95. 258-262.

Dogs were provided with mucosal septal pouches of the fundic stomach and of the duodenal bulb. Acid secretion was stimulated by intravenous infusion of submaximal doses of porcine gastrin or the choline ester Urecholine. Acid perfusion of bulbar pouches profoundly inhibited acid responses to Urecholine. Bulbar acidification produced little or no reduction in acid secretion induced by Urecholine. Secretion of Urecholine does not release gastrin in the dog but may induce acid secretion, mainly by direct stimulation of the HCl glands. The fact that Urecholine-induced acid secretion was not inhibited in the present experiments is consistent with the hypothesis that the bulbar mechanism does not mediate cholinergic stimulation acting on the parietal cells.

Recent experiments (Sjödín and Nilsson 1974) have shown that intravenous infusion of moderate doses of the stable choline ester Urecholine evokes acid secretion without elevated plasma levels of gastrin. This observation may mean that Urecholine activates the glands mainly by direct cholinergic stimulation. The present study was undertaken to examine whether bulbar acidification influences gastric acid responses to cholinergic stimulation induced by Urecholine.

### Methods

Double septal pouches (Pavlov type) of the fundic stomach and of the duodenal bulb (Andersson and 1961) were constructed in 3 mongrel dogs weighing 15-17 kg. Between operations and before starting the dogs were allowed a period of 3 weeks for recovery.

Experiments were started in the morning after the dogs had been fasted for 18-20 h. Gastric juice was collected from Pavlov pouches in 15 min portions. The volume was measured and the acidity was determined by titration with 0.01 M NaOH, using phenolphthalein as indicator. Before an experiment was started gastric acid responses to graded doses of Urecholine were determined in each dog. From experiments a dose (15 µg/kg/h) of Urecholine was selected which evoked acid responses from the pouches corresponding to 70-80% of the maximal secretion to Urecholine. In each experimental Urecholine was given in a 0.9% solution of NaCl which was infused by a calibrated peristaltic (Harvard Apparatus Co., Dover, Mass.) at a rate of 20 ml per hour. When, after 2 1/2 h infusion, a steady



- (X) L. Secretory responses to gastric in Pouch dogs with and without perfusion of the duodenal bulb with 0.1 N HCl.

Number of dogs	Control hours	Range of secretory levels, $\mu\text{mol}/15 \text{ min}$	Relative S.E. of mean of four 15-min periods (per cent)	Mean secretory rate expressed as percentage of the secretory level during the control hour. Half hour periods following the control hour			
				1	2	3	4
3		0.43-0.31	9	80	10	39	96
3		0.18-0.67	11	18	8	21	70
3		0.29-0.47	11	67	15	23	101
		Mean $\pm$ S.E. of mean		$41 \pm 7$	$11 \pm 7$	$28 \pm 6$	$89 \pm 11$

as had been reached, further perfusions were performed with 0.1 M HCl for 1 h. Following completion of acidification gastric secretion was followed for another hour. Further details of the procedure of perfusion have been given elsewhere (Anderson, Nilsson and Uvnäs 1967).

Secretory buffer the buffer mechanism in each dog was efficient in inhibiting acid secretion: control of experiment was performed using gastric secretion (Anderson and Nilsson 1968) as secretory stimulus. These experiments were carried out in the same way as the experiments with Urecholine. Statistical methods of analysis of variance were used (Gosdorp 1967) for the statistical evaluation of the data.

### Results

Bulbar perfusion with acid profoundly inhibited acid responses to exogenous gastrin in all dogs (Table I). When similar gastric secretory rates were induced by Urecholine in the same dogs, bulbar acidification produced some reduction of the acid output in one dog (A), however the inhibitory pattern was different from that observed in experiments with gastrin where inhibition of acid secretion consistently became most pronounced during the acid half-hour of bulbar acidification. In dog B acidification of the bulb did not significantly influence the acid output from the gastric pouches, whereas in dog C an increase in the acid output was observed following perfusion of the bulbar pouch with acid. These results are presented in Table II.

### Discussion

A series of studies in dogs attempts have been made to reveal the characteristics of the mechanism by which acid in the duodenal bulb inhibits acid secretion. From these results appears that the bulbar mechanism is very effective in inhibiting acid responses to exogenous gastrin (Anderson, Nilsson and Uvnäs 1967; Anderson and Nilsson 1969; Nilsson and Runc 1971; Nilsson 1974; Nilsson 1975 c) or to stimuli causing release of gastrin such as test meals (Anderson and Uvnäs 1961; Anderson and Sjodin 1972; Nilsson 1975 b), food feeding (Nilsson 1968; Anderson and Sjodin 1972; Nilsson 1975) or insulin hypoglycaemia (Nilsson 1969; Anderson and Sjodin 1972). Also in the present study bulbar acidification profoundly inhibited acid responses induced by exogenous gastrin, which shows that the bulbar mechanism was very efficient in inhibiting acid secretion in each

TABLE II Secretory responses to Urecholine in Pavlov pouch dogs with and without pyloric duodenal bulb with 0.1 N HCl.

Dog	Number of expts.	Control hours		Mean secretory rate expressed as percentage of the secretory rate during the control hour. Half hour periods following the control hour			
		Range of secretory levels, meq/15 min	Relative S.E. of mean of four 15-min periods (per cent)	1	2	3	4
A	4	0.37-0.55	5	65	67	96	118
B	4	0.30-0.60	8	112	108	112	104
C	4	0.20-0.38	7	126	154	166	128
Mean $\pm$ S.E. of mean				101 $\pm$ 14	110 $\pm$ 12	125 $\pm$ 15	128 $\pm$ 15

dog. However little or no inhibition occurred in experiments in which acid secretion induced by Urecholine.

In order to understand the significance of the present results the nervous and humoral factors involved in the activation of the HCl glands following cholinergic stimulation must be considered. It is well documented that the HCl glands have to be stimulated both vagally and by gastrin to secrete optimally (Uvnäs 1942, Olbe 1964, Sjödin 1977). Thus, resection of the gastric antrum and the duodenal bulb almost completely abolish acid responses to small doses of insulin (Olbe 1964) or to short periods of sham feeding (Olbe 1964, Sjödin 1972). Under certain conditions of vagal stimulation however it seems as if gastrin plays a minor role in the activation of the HCl glands. For example large doses of insulin (Peyssner and Grossman 1955 Olbe 1964) or prolonged sham feeding (Sjödin 1972) produce acid secretion even after resection of gastrin-releasing tissues. Previous studies show that total acidification abolishes acid responses to small doses of insulin (Nilsson 1969 a) and to short periods of sham feeding (Nilsson 1969 b) whereas acid responses to large doses of insulin (Nilsson 1969 a) or to prolonged sham feeding (Nilsson 1969 b) are only partially inhibited. It was suggested (Nilsson 1969 c) from those observations (Peyssner and Grossman 1955 Olbe 1964 Nilsson 1969 a and b) that the bulbar inhibitory mechanism does not inhibit acid responses to stimulation under conditions in which the parietal cells are subjected to a strong direct cholinergic excitation.

The stable choline ester Urecholine, in the doses used in the present study evokes considerable acid responses in Pavlov pouch dogs without significantly raising the plasma gastrin concentration (Sjödin and Nilsson 1974). The mechanism by which Urecholine induces acid secretion is not fully understood. Possibly basal levels of gastrin may act synergistically with Urecholine stimulation on the parietal cells. Alternatively Urecholine may act essentially directly on the HCl glands, independent of gastrin. Urecholine may act through some other as yet unidentified mechanism.

Since the bulbar mechanism is very efficient in inhibiting acid responses to gastrin, and since gastrin levels are not elevated by stimulation with moderate doses of Urecholine, one would expect that Urecholine-induced acid secretion would be profoundly inhibited if gastrin played a significant role in the activation of the HCl glands during Urecholine stimulation. However if Urecholine mainly induces acid secretion by a direct cholinergic

tion of the parietal cells, the present results support the previously proposed hypothesis (Nilsson 1969 c) that the bulbar mechanism does not interfere with direct cholinergic stimulation of the acid secreting glands.

The possible physiological role of the mechanism in the duodenal bulb inhibiting acid secretion may be to contribute to the suppression of postprandial acid secretion and to acid secretion that may occur from the empty stomach during interdigestive periods.

Presence of such a mechanism has been indicated not only in dogs but also in man (Nilsson and Duthie 1965 Wormley 1970) and in the rat (Lundberg and Andersson 1974).

Some of the present and previous (Nilsson 1969 a and b) results suggesting that the bulbar mechanism does not influence direct cholinergic stimulation of the parietal cells,

may be pertinent to discuss the role the bulbar mechanism may play in inhibiting acid secretion in patients with duodenal ulcer disease. It has been suggested that vagal hyperstimulation causes gastric hypersecretion in duodenal ulcer disease (Dragstedt 1942). If such

stimulation of the parietal cells predominates in this disease, inhibitory influences arising from acidification of the bulbar mucosa may be less effective in reducing acid secretion in

duodenal ulcer patient than in normal man.

This study was supported by research grants from Statens Medicinska Forskningsråd (3521) and from Svenska Läkaresällskapet. The author is grateful to Miss Gunilla Tholin, Miss Elisabeth Torén and Miss Sara Lennqvist for technical and secretarial assistance.

# References

1. NILSSON, S. and G. NILSSON, pH-dependence of the mechanism in the duodenal bulb inhibiting gastric acid responses to exogenous gastric Acids. *Physiol. scand.* 1969 76, 183-190.
2. NILSSON, S. and L. SÄCKO, Inhibition of gastric secretion by acidification of the duodenal bulb before and after transection of the pylorus. *Scand. J. Gastroenterol.* 1972, 7, 131-136.
3. NILSSON, S. and B. UVELL, Inhibition of postprandial gastric secretion by Pavlov pouches by instillation of hydrochloric acid into the duodenal bulb. *Gastroenterology* 1961 41, 436-440.
4. NILSSON, S., G. NILSSON and B. UVELL, Effect of acid in proximal and distal duodenal pouches on gastric secretory responses to gastric and barostat. *Acta physiol. scand.* 1967 71, 368-378.
5. JACOBSON, L. R. Pathogenesis of gastroduodenal ulcer. *Arch. Surg.* 1942, 44, 438-450.
6. NILSSON, S. and H. L. DUTHIE, Inhibition of gastric secretion in the human stomach. *Lancet* 1965, 2, 1012-1016.
7. ANDERSSON, S. and S. ANDERSSON, The role of the upper duodenum in controlling gastric acid secretion in the rat. *Scand. J. Gastroenterol.* 1974, 9, 623-627.
8. LARSEN, G. Effect of acid in the duodenal bulb on gastric secretory responses to histamine hypoglycemia. *Acta physiol. scand.* 1968 73, 476-483.
9. LARSEN, G. Effect of acid in the duodenal bulb on gastric secretory responses to sham feeding. *Acta physiol. scand.* 1969 b, 77, 308-315.
10. LARSEN, G. Studies of the mechanism by which acid in the duodenal bulb inhibits gastric acid secretion. Dissertation, Stockholm, 1969 a.
11. LARSEN, G. Effect of acidification of totally denervated pouches of the duodenal bulb on gastric acid secretion in dog. *Acta physiol. scand.* 1974, 92, 568-571.
12. LARSEN, G. Increased plasma gastrin levels in connection with inhibition of gastric acid responses to sham feeding following bulbar perfusion with acid in dogs. *Scand. J. Gastroenterol.* 1975 a, 10, 273-278.
13. LARSEN, G. Effect of acid in the duodenal bulb on acid and pepsin responses to test meal in dog. *Scand. J. Gastroenterol.* 1975 b, 10, 279-283.
14. LARSEN, G. Effect of bulbar perfusion with acid, hypertonic solutions and acetylcholine on gastric acid secretion in dog. *Scand. J. Gastroenterol.* 1975 c, 10, 284-288.

TABLE II Secretory responses to Urecholine in Pavlov pouch dogs with and without pyloric duodenal bulb with 0.1 N HCl.

Dog	Number of expts.	Control hours		Mean secretory rate expressed percentage of the secretory rate during the control hour. Six periods following the control		
		Range of secretory levels, mcg/15 min	Relative S.E. of mean of four 15-min periods (per cent)	1	2	3
A	4	0.37-0.55	5	65	67	96
B	4	0.30-0.60	8	112	108	112
C	4	0.20-0.38	7	126	154	166
Mean $\pm$ S.E. of mean				101 $\pm$ 14	110 $\pm$ 13	125 $\pm$ 13

dog. However little or no inhibition occurred in experiments in which acid secretion was induced by Urecholine.

In order to understand the significance of the present results the nervous and humoral factors involved in the activation of the HCl glands following cholinergic stimulation must be considered. It is well documented that the HCl glands have to be stimulated both by gastrin and by vagal stimulation (Uvnäs 1942, Olbe 1964, Sjödin 1972). Thus, removal of the gastric antrum and the duodenal bulb almost completely abolishes acid responses to small doses of insulin (Olbe 1964) or to short periods of sham feeding (Olbe 1964, Sjödin 1972). Under certain conditions of vagal stimulation, however, it seems as if gastrin plays a minor role in the activation of the HCl glands. For example large doses of insulin (Pevsner and Grossman 1955, Olbe 1964) or prolonged sham feeding (Sjödin 1972) produce acid secretion even after resection of gastrin-releasing tissues. Previous studies show that acidification abolishes acid responses to small doses of insulin (Nilsson 1969 a) or to short periods of sham feeding (Nilsson 1969 b), whereas acid responses to large doses of insulin (Nilsson 1969 a) or to prolonged sham feeding (Nilsson 1969 b) are only slightly inhibited. It was suggested (Nilsson 1969 c) from those observations (Pevsner and Grossman 1955, Olbe 1964, Nilsson 1969 a and b) that the bulbar inhibitory mechanism does not inhibit acid responses to stimulation under conditions in which the parietal cells are subjected to a strong direct cholinergic excitation.

The stable choline ester Urecholine, in the doses used in the present study evokes considerable acid responses in Pavlov pouch dogs without significantly raising the plasma gastrin concentration (Sjödin and Nilsson 1974). The mechanism by which Urecholine induces acid secretion is not fully understood. Possibly basal levels of gastrin act synergistically with Urecholine stimulation on the parietal cells. Alternatively Urecholine may act essentially directly on the HCl glands, independent of gastrin. Urecholine may act through some other as yet unidentified mechanism.

Since the bulbar mechanism is very efficient in inhibiting acid responses to gastrin, since gastrin levels are not elevated by stimulation with moderate doses of Urecholine, one would expect that Urecholine-induced acid secretion would be profoundly inhibited if gastrin played a significant role in the activation of the HCl glands during Urecholine stimulation. However if Urecholine mainly induces acid secretion by a direct cholinergic

## Relative Contribution of Superficially Bound and Extracellular Calcium to Activation of Contraction in Isolated Rat Portal Vein

By

STEFAN B. SJÖGREN, BENGT UVELLUS and BÖRJE JOHANSSON

Received 8 April 1975

### Abstract

JOHANSSON, S. B., UVELLUS, B. and JOHANSSON, B. *Relative contribution of superficially bound and extracellular calcium to activation of contraction in isolated rat portal vein.* Acta physiol. scand. 1975. 95. 263-269

Spontaneous electrical and mechanical activity of the isolated rat portal vein is abolished after only 3 min in essentially Ca-free medium, and after 3-6 min there is no contractile response to depolarizing (2 mM K<sup>+</sup>), Ca-free solution. In the present study we have examined the electrical and mechanical responses of the portal vein to depolarization with membrane-permeable analogues of Ca<sup>2+</sup> (2.5 mM) after periods of variable length in Ca-free standard solution. After 30 to 60 min of Ca depletion, slow contraction occurred in response to the high-K<sup>+</sup> solution with 2.5 mM Ca<sup>2+</sup>. When the period in Ca-free medium was reduced below 30 min an early, faster phase appeared in the contractile response, and this phase was even faster the shorter the time of Ca depletion. It is suggested that the slow contraction obtained after 3 min or more time in Ca-free solution is due to release of extracellular Ca for activation and that the faster phase seen after shorter periods of Ca depletion is due to release of superficially bound Ca. This latter pool of tissue bound Ca does not alone produce contraction in response to depolarization, suggesting that extracellular Ca is required to trigger the release perhaps through regenerative process.

It is generally agreed that Ca<sup>2+</sup> is necessary for contraction in smooth muscle as well as in other contractile systems. However, the relative importance of different possible sources of activator calcium in smooth muscle is still a matter of debate. With regard to vascular smooth muscle it has been found that certain preparations of isolated blood vessels, e.g. the rabbit aorta, maintain their ability to contract in response to various stimulants even after quite long periods in calcium-free solutions (Bohr 1963). This could indicate that in these vessels tissue bound Ca may be released and utilized for contraction. On the other hand, there are other preparations of vascular smooth muscle such as the rat portal vein, which lose their responsiveness after only a few minutes in Ca<sup>2+</sup>-free solution (e.g. Johansson 1974, see also below). It appears therefore that activator calcium in these tissues is more intimately related to extracellular Ca, but the engagement of bound fractions can by no means be excluded.

- NILSSON, G. and S. J. RUNC, Failure of high  $pCO_2$  in the duodenal bulb in labetal gastric acid secretion. *Acta physiol. scand.* 1971 *81* 349-354.
- OLSSON, L., Effect of resection of gastrin releasing regions on acid response to sham feeding and on hypoglycemia in Pavlov pouch dogs. *Acta physiol. scand.* 1964, *62*, 169-175.
- PEVNER, L. and M. L. GROSSMAN, The mechanism of vagal stimulation on gastric acid secretion. *Gastroenterology* 1955 *28* 493-499.
- SJÖÖHN, L., Potentiation of the gastric secretory response to sham feeding in dogs by infusion of p- and pentagastrin. *Acta physiol. scand.* 1972, *85* 24-32.
- SJÖÖHN, L. and G. NILSSON, Plasma gastrin levels and gastric acid secretion in dogs during stimulation of Urecholine. *Scand. J. Gastroent.* 1974 *9* 747-750.
- SNEDECOR, G. W. and W. G. COCHRAN, *Statistical methods*. Iowa State Press, Ames Iowa, 1962.
- UJVALA, B., The part played by the pyloric region in the cephalic phase of gastric secretion. *Acta physiol. scand.* 1942, *4* suppl. 13.
- WOMERSLEY, K. G. Response to duodenal acidification on man II. Effects on the gastric secretory response to pentagastrin. *Scand. J. Gastroent.* 1970, *5* 207-213.

## Relative Contribution of Superficially Bound and Extracellular Calcium to Activation of Contraction in Isolated Rat Portal Vein

By

STEFAN B. JOHANSSON, BENGT UVELLUS and BORJE JOHANSSON

Received 8 April 1975

### Abstract

JOHANSSON, S. B., UVELLUS, B. and JOHANSSON, B. *Relative contribution of superficially bound and extracellular calcium to activation of contraction in isolated rat portal vein.* Acta physiol. scand. 1975. 95. 263-269

Spontaneous electrical and mechanical activity of the isolated rat portal vein is abolished after only 1 min in essentially Ca-free medium, and after 5-6 min there is no contractile response to depolarizing 2 mM  $K^+$ , Ca-free solution. In the present study we have examined the electrical and mechanical phases of the portal vein to depolarization with simultaneous readjustment of  $Ca^{++}$  (2.5 mM) after periods of variable length in Ca-free standard solution. After 10 to 60 min of Ca depletion, slow contracture occurred in response to the high- $K^+$  solution with 2.5 mM  $Ca^{++}$ . When the period in Ca-free medium is reduced below 30 min an early faster phase appeared in the contracture response, and this phase was even rapid the shorter the time of Ca depletion. It is suggested that the slow contracture obtained after 1 min or more was mainly extracellular Ca for activation and that the faster phase seen after shorter periods of Ca depletion is due to release of superficially bound Ca. This latter pool of tissue bound Ca does at least produce contraction in response to depolarization, suggesting that extracellular Ca is required to trigger the release perhaps through regenerative processes.

It is generally agreed that  $Ca^{++}$  is necessary for contraction in smooth muscle as well as in other contractile systems. However, the relative importance of different possible sources of activator calcium in smooth muscle is still a matter of debate. With regard to vascular smooth muscle it has been found that certain preparations of isolated blood vessels, e.g. the rabbit aorta, maintain their ability to contract in response to various stimulants even after quite long periods in calcium-free solutions (Bohr 1963). This could indicate that in these vessels tissue bound Ca may be released and utilized for contraction. On the other hand, there are other preparations of vascular smooth muscle such as the rat portal vein, which lose their responsiveness after only a few minutes in  $Ca^{++}$ -free solution (e.g. Johansson 1974 see also below). It appears therefore that activator calcium in these tissues is more intimately related to extracellular Ca, but the engagement of bound fractions can by no means be excluded.

The present study is an attempt to obtain information on the different sources of  $\text{Ca}^{2+}$  in portal vein. Some of the results have been reported in preliminary form (Uvelius, Sigurdsson and Johansson 1974).

### Materials and methods

Experiments were performed on 40 portal vein preparations from rats of the Sprague-Dawley strain weighing 200–300 g. The animals were killed by a blow on the neck and the portal veins were dissected out. Experiments in which only isometric tension was recorded, the length of the preparations was about 1 mm. These were mounted in a 90 ml mantled organ bath and connected to a Grass FT03 force transducer. A passive tension of about  $4 \cdot 10^{-6}$  N. The experiments with simultaneous recording of electrical and mechanical activity were carried out with the sucrose gap apparatus using longer preparations of pre-mesenteric vein (see Axelsson *et al.* 1967). The electrical signals were amplified and recorded as described by Johansson and Mellander (1975).

The bathing medium was a standard Tris-buffered solution ("Na-tris") of the following composition: mM NaCl 120, KCl 6.0,  $\text{MgCl}_2$  1.0, glucose 11.5, tris(hydroxymethyl)aminomethane (Tris; see Sigurdsson 1974) 30. The pH was titrated to 7.4 with 1 M HCl. The temperature in the bath was held at 37°C. To this normally  $\text{Ca}^{2+}$ -free solution various amounts of  $\text{CaCl}_2$  were added giving  $\text{Ca}^{2+}$  concentrations 0.5, 1.0, 2.5 and 5.0 mM respectively. The depolarizing solutions ("K-tris") had the same composition as above, except that all NaCl was substituted with equimolar amounts of KCl. All solutions were bubbled with 100%  $\text{O}_2$ .

Before the actual experiment started the muscles were allowed to accommodate for at least 60 min in Na-tris with 2.5 mM  $\text{Ca}^{2+}$ .

### Results

Fig. 1 A shows the spontaneous isometric contractions (upper tracing) and an AC sucrose gap recording of electrical activity (lower tracing) from a rat portal vein in "normal" solution. After the one hour accommodation period, this superfusion medium was replaced by  $\text{Ca}^{2+}$ -free Na-tris solution. Electrical and mechanical activity then rapidly disappeared. Six min later (Fig. 1 B) the muscle was transferred to K-tris solution with 2.5 mM  $\text{Ca}^{2+}$ . This caused an early fast increase in tension associated with the appearance of electrical spike activity. When the contraction reached near maximum the spikes decreased in amplitude and number and finally disappeared. The later sustained contracture was associated with maintained membrane depolarization (not seen in the present AC recording). When the time in Na-tris  $\text{Ca}^{2+}$ -free solution preceding the switch to K-tris with 2.5 mM  $\text{Ca}^{2+}$  was increased to 10 min (Fig. 1 C) and 20 min (Fig. 1 D), respectively, the rate of rise of the contractile force became successively slower although the final contracture amplitude was unaltered. After 10 min only few and after 20 min no spikes were seen.

The differences in time course of K<sup>+</sup> contractures due to different durations of the preceding  $\text{Ca}^{2+}$  depletion were studied systematically in mechanical experiments with recording of isometric force. Original recordings from one such experiment are exemplified in Fig. 2. When the muscle, after 30 min in Na-tris  $\text{Ca}^{2+}$ -free solution was placed in K-tris with 2.5 mM  $\text{Ca}^{2+}$  (Fig. 2 A), a slowly developing contracture of the same type as in Fig. 1 D was obtained. On the other hand, when the muscle was transferred to the depolarizing solution already after 6 min, the development of tension was much faster although the final tension amplitude was unchanged (Fig. 2 B). If  $\text{Ca}^{2+}$ -free depolarizing solution was administered after 6 min



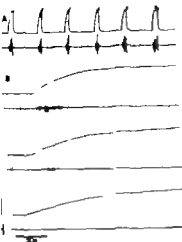


Fig. 1

A. Normal spontaneous activity of the rat portal vein recorded in sucrose gap apparatus. Upper mechanical activity, lower tracing electrical activity (AC-recording, see text). B-D. Effect of K-tris 3 mM (B), 10 mM (C) and 20 mM (D) in Ca-free solution.

Mechanical response to K-tris with 2.5 mM Ca after the muscle had been in Ca-free Na-tris for (A) Response to K-tris with 3 mM Ca (B) and to K-tris with 0 mM Ca (C) after 6 min in Ca-free solution.



Fig. 2

in Na-tris no development of tension was seen (Fig. 2 C). Thus depolarization without previous readministration of calcium failed to produce contraction already after short periods of preceding Ca depletion.

A diagram in Fig. 3 summarizes the differences in time course of responses to K-tris with 2.5 mM  $\text{Ca}^{++}$  after 6, 10, 15, 30 and 60 min of calcium depletion as obtained in muscles that had previously accommodated in Na-tris with 2.5 mM  $\text{Ca}^{++}$  for 30 min. Tension as a function of time is expressed in per cent of the ultimate contracture level for each individual

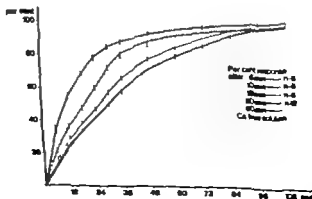


Fig. 3. Comparison of the time course of contractile responses elicited by K-tris with 2.5 mM  $\text{Ca}^{++}$  after various times in Ca-free Na-tris solution. Contraction commenced after 120 ms and is set to 100%. Vertical bars represent S.E.

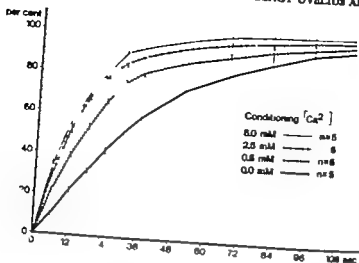


Fig. 4. Comparison of course of contraction in K-tris with 2.5 mM Ca which had accommodated to various concentrations. Before the test the muscle was in Na-tris solution for 6 min. Contraction amplitude after set to 100%. Vertical bars represent S.E.

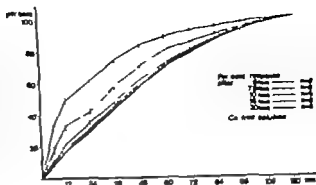
preparation. As is seen in the figure, those muscles which had been in Ca-free Na-tris for 30 and 60 min, respectively, developed tension at the same slow rate. Those which had been in this solution for lesser periods showed a faster increase, steeper the shorter the period in Ca-free solution.

A separate series of experiments was performed in order to study the influence of the  $\text{Ca}^{2+}$  concentration during the accommodation period on the contractile response after a given time of Ca depletion. The muscles were allowed to accommodate for 30 min in Na-tris solution but now with the following concentrations of Ca: 0, 0.5, 2.5 and 5.0 mM. After that period they were placed in Ca-free solution for 6 min and then exposed to K-tris with 2.5 mM Ca. The results are summarized in Table 1. It is seen that as the Ca concentration in the accommodating solution was increased from 0 to 2.5 mM a faster tension development occurred in the K-tris 2.5 mM Ca solution. A further increase of Ca in the accommodating solution to 5 mM had no additional effect.

The results shown in Fig. 3 and 4 indicated that the fast component of the contractile response was dependent on Ca accumulated in the preparation during the previous accommodation period. To study whether such accumulation could occur also in the polarized state the following experiments were performed. The muscles were placed in Na-tris for 30 min in Na-tris with 2.5 mM Ca. They were then transferred to Ca-free Na-tris for 30 min followed by exposure to K-tris with 2.5 mM Ca for 4 min. Thereafter they were placed for various periods (6, 7.5, 10, 15 and 30 min) in Ca-free Na-tris solution where they rapidly relaxed. They were then allowed to contract again in K-tris with 2.5 mM Ca. The results are shown in Fig. 5. Variations in the time course of the second contracture response were expected to reflect differences in the loss of calcium which had possibly accumulated during the first contracture period. No significant difference in time course is seen between the curves for 10, 15 and 30 min depletion but the results after 7.5 and 6 min show that a fast phase occurs also under the conditions of these experiments.

### Discussion

Spontaneous electrical and mechanical activity in the isolated portal vein is abolished after only 2–3 min in nominally Ca-free solution (e.g. Johansson 1974) and the contractile



to  $K$ -depolarization without simultaneous administration of exogenous  $Ca^{++}$  disappears after 5-6 min (Fig. 2). This implies that the concentration of free calcium in the extracellular space decreases rapidly in the nominally  $Ca$ -free medium since earlier studies have shown that spontaneous activity and  $K$  contractions require threshold concentrations of 5 and 0.1 mM  $Ca^{++}$  respectively (Uvelius, Sigurdsson and Johansson 1975, Blamire and Johansson 1970). The difference in time course of the contractile responses to  $K$ -tris with 5 mM  $Ca^{++}$  after (for instance 6 and 30 min of  $Ca$  depletion (Fig. 1 and 2) cannot therefore be due to differences in the amount of free  $Ca$  remaining in the extracellular fluid. It is more likely that a superficially bound  $Ca$  fraction is mobilized after the short periods of depletion and that it contributes to the contractile activation and to the spike phenomenon which accompanies the fast phase of the contraction. Calcium in the extracellular fluid is usually necessary to trigger the release of this bound fraction at the depolarization of the cell, after 6 min of depletion,  $[Ca^{++}]_o$  has dropped below the level required (Fig. 2 C). Within 6 to 30 min of preceding  $Ca$  depletion the response to  $K$  depolarization with simultaneous administration of exogenous  $Ca^{++}$  appears to be due to a combined release of  $Ca$  from the superficial bound pool (fast phase of the response) and influx of extracellular  $Ca^{++}$  (slow phase). The identical shape of the responses after 30 and 60 min defines the slow phase. The reasons for believing that this phase represents influx of extracellular  $Ca$  are that its amplitude is greatly dependent on  $[Ca^{++}]_o$  and that it does not change even with prolonged exposure to  $Ca$ -free solution (Blamire and Johansson 1970, Hellstrand, Johansson and Ingberg 1972, Uvelius *et al.* 1975). The portal vein undoubtedly contains deeper cellular pools of  $Ca$  which seem very resistant to depletion and which, in the absence of extracellular  $Ca$ , can support contraction only when exceptional modes of stimulation are applied (Andersson *et al.* 1974).

The amount of calcium which can be liberated from the superficial binding sites to give the fast phase of the contraction varies not only with the time in the  $Ca$ -depleting medium (Fig. 3) but also with the  $[Ca^{++}]_o$  in the preceding accommodation period (Fig. 4). It appears that this pool is practically saturated at normal levels of  $[Ca^{++}]_o$ . Furthermore, the results shown in Fig. 5 indicate that the superficial binding sites can accumulate calcium also when the cell is depolarized. The anatomical localization of these sites remains to be demonstrated.

but the basal membrane, the plasma membrane and perhaps the superficially located plasmic reticulum (Devine, Somlyo and Somlyo 1973) appear most likely.

Other authors have previously distinguished between slow and rapid component vascular responses to various stimuli and have given different interpretations to the findings. Bohr (1963) in a study of epinephrine stimulated rabbit aortic strips attributed delay in the fast and the slow phase of the contractile response to effects on membrane excitation and on excitation-contraction coupling, respectively. More recently Sitrin and Blair (1970) have ascribed fast and slow components in the responses of rabbit aortic and dog mesenteric artery strips to different sources of activator calcium. In a study of rabbit mesenteric portal vein Collins, Sutter and Telser (1971) found two components in the response to noradrenaline and potassium and suggested that the early phase was related to release of superficially bound calcium and the later one to influx of extracellular calcium. A constrictor response of the isolated rabbit ear artery also shows a distinct rapid component followed by a slow phase. The first component was attributed by Bevan and Waterson (1971) to myogenic spread of excitation from the surface layer of smooth muscle cells and to the second one related to saturation of the extracellular space with the stimulant (norepinephrine). Steinsland, Furchgott and Kirpekar (1973), on the other hand, considered the biphasic response of the rabbit ear artery to reflect different sources of activator calcium. Evidence has been presented indicating that different stimuli (e.g. noradrenaline and potassium) may differ in their relative utilization of different calcium pools in vascular smooth muscle (e.g. Peiper, Griebel and Wende 1971; van Broemen *et al.* 1971; Golenhofen, Hermstein and Lammel 1973).

It is somewhat difficult to make a direct comparison between our own results and those of earlier investigators mentioned above due to the different preparations and modes of stimulation. Our own results and interpretations are in good agreement with those of Blair and Bohr (1971), Collins *et al.* (1971) and Steinsland *et al.* (1973). The new point of our findings is the indication that extracellular Ca is required for release of Ca from the superficial binding sites perhaps through a regenerative mechanism. This is suggested by the fact that this pool contains effective amounts of Ca after more than 15 min in Ca-free solution (Fig. 3), but that it already after 6 min has lost its ability to alone support contractile responses to depolarization.

The present study was supported by grants from the Swedish Medical Research Council (23-14) to the Medical Faculty, University of Lund, and from AB Hålsjö, Göteborg. Thanks are due to Mrs. Åke Lundahl and Mrs. Monica Hedenhjem for able technical assistance.

## References

- ANDERSSON, C., P. HALLSTRAND, B. JOHANSSON and A. RIMBERG. Contraction in vascular smooth muscle induced by hypertonicity. Calcium dependence and mechanical characteristics. *Acta physiol. Scand.* 1974, 90: 451-461.
- AXELSSON, J., B. WAHLSTRÖM, B. JOHANSSON and O. JOHANSSON. Influence of the ionic environment on spontaneous electrical and mechanical activity of the rat portal vein. *Circulat. Res.* 1967, 21: 609-618.
- BEVAN, J. A. and J. G. WATERSON. Biphasic constrictor response of the rabbit ear artery. *Circulat. Res.* 1971, 28: 655-661.

10. G. and B. JONASSON, Effects of calcium and sodium on contracture tension in the smooth muscle of rat portal vein. *Pflügers Arch. ges. Physiol.* 1970. 321 143-153.
11. D. F. Vascular smooth muscle: Dual effect of calcium. *Science* 1963. 139 397-399.
12. G. A., M. C. SUTLER and J. C. TIGER, Calcium and contractions in the rabbit anterior mesenteric artery. *Canad. J. Physiol. Pharmacol.* 1971. 30. 289-299.
13. C. E., A. V. SOMLYO and A. P. SOMLYO, Sarcoplasmic reticulum and mitochondria as calcium sequestration sites in smooth muscle. *Phil. Trans. R.* 1973. 163. 17-23.
14. OTSU, K., M. HARMOTEN and E. LAMERT, Membrane potential and contraction of vascular smooth muscle (portal vein) during application of noradrenaline and high potassium, and selective inhibitory effects of Iproveratril (Verapamil). *Microvasc. Res.* 1973. 5 73-80.
15. FRANK, P., B. JONASSON and A. RIVEROLO, Influence of extracellular calcium on isometric force velocity of shortening in depolarized venous smooth muscle. *Acta physiol. scand.* 1972. 84. 523-537.
16. SMITH, R., Determinants of vascular reactivity. *Fed. Proc.* 1974. 33. 121-126.
17. SMITH, R. and S. MILLANDER, Steady and dynamic components in the vascular myogenic response to flow changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Microvasc. Res.* 1973. 36. 74-83.
18. U., L. GRENEL and W. WIDIG, Activation of vascular smooth muscle of rat aorta by noradrenaline depolarization: Two different mechanisms. *Pflügers Arch. ges. Physiol.* 1971. 330. 74-89.
19. J. M. D. and D. F. BOWEN, Ca and Na interaction in vascular smooth muscle contraction. *Amer. J. med.* 1971. 230. 1124-1128.
20. KAWO, O. S., R. F. FURCHGOTT and S. M. KIRKPATRICK, Epinephrine vasoconstriction of the rabbit coronary. *Circulat. Res.* 1973. 32. 40-58.
21. KILB, B. S. R. SOUTHWORTH and B. JONASSON, Role of superficially bound Ca for potassium contraction in rat portal vein. *Acta physiol. scand.* 1974. 91 2 A.
22. KILB, B. S. R. SOUTHWORTH and B. JONASSON, Strontium and barium as substitutes for calcium on electrical and mechanical activity in rat portal vein. *Blood Vessels* 1974. 11 245-253.
23. BRIDGEMAN, C., B. R. FARHAM, P. GERRA and E. D. McNAUGHTON, Contraction coupling in rabbit is studied by the bromocaine method for measuring cellular calcium influx. *Circulat. Res.* 1972. 30. 34.

## Inhibition of Induced Pinocytosis in *Amoeba proteus* by Membrane Stabilizing Drugs

By

J.-O. JOSEFSSON, G. JOHANSSON and S. ELISABETH HANSSON

Received 16 April 1975

### Abstract

JOSEFSSON, J.-O., G. JOHANSSON and S. ELISABETH HANSSON. Inhibition of induced pinocytosis in *Amoeba proteus* by membrane stabilizing drugs. Acta physiol. scand. 1975, 95, 270-285.

The effect of membrane stabilizing drugs on cation induced pinocytosis was studied in *Amoeba proteus*. Initially the presence of local anesthetic drugs during a pinocytosis cycle had a stimulating effect on its formation, however the capacity to develop pinocytotic channels was reversibly inhibited after a pretreatment with these drugs. Imipramine, tetracaine and the phenothiazines had effects similar to anaesthetics. The local anaesthetics inhibited pinocytosis in the following order: dibucaine > tetracaine > bupivacaine > lidocaine > procaine, and the phenothiazines: thioridazine > prochlorperazine > chlorpromazine > promethazine. Pinocytosis, when induced by Na<sup>+</sup> or K<sup>+</sup>, was more affected by the drug calcium binding agents than pinocytosis induced by Ca<sup>++</sup>. After pretreatment with inhibitory concentrations of dibucaine ( $3 \cdot 10^{-4}$  M) the depolarization of the membrane and the conductance increase during pinocytosis were normal, while the increase of oxygen uptake during the pinocytosis cycle was abolished. Addition of Ca<sup>++</sup> before, during or after dibucaine treatment decreased the effect of the drug. Conversely, in Ca<sup>++</sup>-treated cells, cation induced pinocytosis was less inhibited by Ca<sup>++</sup> than pinocytosis in normal cells. Addition of EGTA to the inducing solutions potentiated the inhibitory effect of the drug. It was concluded that these drugs release Ca<sup>++</sup> from the cell surface and that higher concentration or after prolonged action time interferes with a Ca<sup>++</sup> mechanism which couples the membrane and contractile systems in the plasma membrane.

The pinocytosis mechanism in *Amoeba proteus* has been suggested to be an excitation-contraction process in which Ca<sup>++</sup> couples the excitation (depolarization) of the membrane to a channel forming system in the cytoplasm (Josefsson 1968). On the other hand, Ca<sup>++</sup> producing increased membrane resistance and increased membrane potential, inhibit the induction of pinocytosis. Monovalent inorganic cations have a Ca<sup>++</sup> dependent depolarizing action which parallels their ability to induce invagination of the cell membrane. (Brannstrom & Freeman 1967, Josefsson *et al.* 1975). In the past years an important and specific drug class, agents characterized pharmacologically as membrane stabilizers has been elucidated. These agents interfere with physiological functions which are dependent on the movement of Ca<sup>++</sup>, consequently in an excitation-contraction mechanism the local anaesthetics may interfere with the coupling between excitation and contraction (Feinstein 1974, Feinstein and

in 1966 a) as well as with the initial excitatory event (which in amoeba is  $\text{Ca}^{++}$  dependent), the present report the inhibiting effect of a number of membrane stabilizing drugs on the pmoctoses induced by  $\text{K}^{+}$  and this is described and an attempt is made to define the site of action of these drugs.

### Methods

*Amoeba proteus*, fed on *Tetrahymena pyrriformis*, were starved in Pringheim solution three days before an experiment. The composition of the culture medium (Pringheim) was (mM):  $\text{Na}^{+}$  0.22,  $\text{K}^{+}$  0.35,  $\text{Ca}^{++}$  0.1,  $\text{Mg}^{++}$  0.08,  $\text{Fe}^{++}$  0.007,  $\text{HPO}_4^{--}$  0.12,  $\text{Cl}^{-}$  0.35,  $\text{NO}_3^{-}$  1.70,  $\text{SO}_4^{--}$  0.09 pH 7.0. All studies were made at room temperature (22–25°C). Inducing salts were chlorides of Analaar grade obtained from the British Drug Houses, Poole, England. Distilled water was used throughout the experiments.

Determination of pmoctoses was made at 23°C (temperature being measured on the microscope stage) according to modification of technique by Josephson (1968). Exchange of extracellular medium by the inducing solution was made with the amoebae (200–400) on glass microscope slides. The total volume used was 2.5 ml and the exchange was carried out in three steps and completed within one minute. The efficacy of exchange was calculated from deletion of 3-O-methyl-D-glucose (methyl  $^3\text{C}$ ) to be better than  $1 \cdot 10^4$  each amoeba was then expected for pmoctocytic channels during 30 sec only. This procedure was continued until 40 amoebae had been observed. Dividing the sum of the number of channels by 40 (the number of observations) gives the mean value of channels observed in an average amoeba during the first 20 minutes of the pmoctocytic cycle. This value is designated channels in the graphs and used as an index of pmoctocytic activity.

Preincubation of cells with drugs was carried out in moist chambers. The cells were adherent to microscope slides and the culture solution was exchanged for the solution containing the drug. The drugs were applied either dissolved in distilled water, tris buffer or in Pringheim solution from which divalent cations had been omitted (CFP = calcium free Pringheim). Cells used as controls were preincubated in CFP solution (0.11 mM  $\text{Na}_2\text{HPO}_4$  and 0.35 mM  $\text{KCl}$  at pH 7.0) or in 0.3 mM  $\text{NaCl}$ . The salts used were of analytical grade, pH was adjusted with  $\text{HCl}$  or  $\text{LiOH}$  and determined with an expanded scale electro-meter using Radiometer electrodes.

Salts of EGTA (L3-bis 2-amino-ethoxyethane tetraacetic acid) were used at pH 7.0 to obtain extracellular media of low  $\text{Ca}^{++}$  concentrations. A conditional formation constant of the  $\text{Ca}$ -EGTA complex (pH 7.0, ionic strength 0.1 and 20°C) was calculated to be  $10^9$  from  $\log K_{\text{CaEGTA}} = 11.0$  and dissociation constants for the chelator given by Schirmer and Schirmer (1960). The concentrations of the free ionized  $\text{Ca}^{++}$  was calculated according to the formula

$$p\text{Ca} = 6.7 + \log \left\{ \frac{[\text{EGTA added}]}{[\text{CaCl}_2 \text{ added}] - 1} \right\}$$

Respiratory rates in cell populations were measured at 22°C with Clark-type polarographic electrode held inside plunger which fitted tightly into 6 ml sample chamber equipped with magnet bar. The percent oxygen consumed was measured with an YSI model II oxygen monitor (Yellow Springs Instrument Co. USA) and recorded on strip chart recorder (Servogor Goertz, Austria). Aliquots containing about 30 000 cells were taken from cell suspensions spiked with magnetic starter. The spiked suspension was centrifuged at low speed (300 g/min) in order to separate the cells and incubated with the drug solution. After 30 min of incubation with the drug, the cells were carefully washed with Pringheim solution before they were transferred to the electrode chamber. The oxygen consumption was registered in arbitrary units from the slope of the graphs. Measurements were started when the cells had been in the solution for 5 min. Measurements of oxygen consumption were also made manually on single cells with the cartesian driver technique according to the detailed description by Harrison *et al.* (1968).

The calcium content of cells and external media was measured with Unicam SP 90 Atomic absorption spectrophotometer. Cells are homogenized with ultrasound (Branson Sonifier) and  $\text{LaCl}_3$  (final concentration 0.5 M) is added to overcome the suppression of the calcium absorption by organic material. To separate the extracellular calcium from the cellular calcium the cell suspension was washed in the centrifuge with 1 M  $\text{NaCl}$  pH 6.0 and incubated at room temperature for 30 min with 1 mM  $\text{LaCl}_3$ . The calcium content of the extracellular solution so obtained was taken as measure of the calcium bound outside.

## Inhibition of Induced Pinocytosis in *Amoeba proteus* by Membrane Stabilizing Drugs

By

J-O JOSEFSSON, G JOHANSSON and S. ELIZABETH HANSSON

Received 16 April 1975

---

### Abstract

JOSEFSSON J-O., G JOHANSSON and S ELIZABETH HANSSON *Inhibition of induced pinocytosis in *Amoeba proteus* by membrane stabilizing drugs.* Acta physiol. scand. 1975, 95, 270-285.

The effect of membrane stabilizing drugs on cation induced pinocytosis was studied in *Amoeba proteus*. Initially the presence of local anesthetic drugs during a pinocytosis cycle had a stimulating effect on clathrate formation, however the capacity to develop pinocytotic channels was reversibly inhibited after a period of treatment with these drugs. Imipramine, vinblastine and the phenothiazines had effects similar to local anesthetics. The local anesthetics inhibited pinocytosis in the following order: dibucaine > tetracaine > bupivacaine > lidocaine > procaine, and the phenothiazines: thioridazine > prochlorperazine > chlorpromazine > promethazine. Pinocytosis, when induced by Na<sup>+</sup> or tris, was more affected by the drugs as calcium binding agents than pinocytosis induced by K<sup>+</sup>. After pretreatment with inhibitory concentrations of dibucaine ( $3 \cdot 10^{-4}$  M) the depolarization of the membrane and the conductance increase during pinocytosis were normal, while the increase of oxygen uptake during the pinocytotic cycle was abolished. Addition of Ca<sup>++</sup> before, during or after dibucaine treatment decreased the effect of the drug. Conversely in untreated cells, cation induced pinocytosis was less inhibited by Ca<sup>++</sup> than pinocytosis in normal cells. Addition of EGTA to the inducing solutions potentiated the inhibitory effect of the drug. It is suggested that these drugs release Ca<sup>++</sup> from the cell surface and at higher concentration or after prolonged incubation interfere with a Ca<sup>++</sup> mechanism which couples the membrane and contractile systems in the cell plasma.

---

The pinocytosis mechanism in *Amoeba proteus* has been suggested to be an excitation-contraction process in which Ca<sup>++</sup> couples the excitation (depolarization) of the membrane to a channel forming system in the cytoplasm (Josefsson 1968). On the other hand excitation producing increased membrane resistance and increased membrane potential, inhibits induction of pinocytosis. Monovalent inorganic cations have a Ca<sup>++</sup> dependent depolarizing action which parallels their ability to induce invagination of the cell membrane. (Brandt & Freeman 1967 Josefsson *et al* 1975) In the past years an important and specific class of agents characterized pharmacologically as membrane stabilizers has been elucidated. These agents interfere with physiological functions which are dependent on the movement of Ca<sup>++</sup> consequently in an excitation-contraction mechanism the local anesthetics may interfere with the coupling between excitation and contraction (Feinstein 1964 Blaustein 1968).



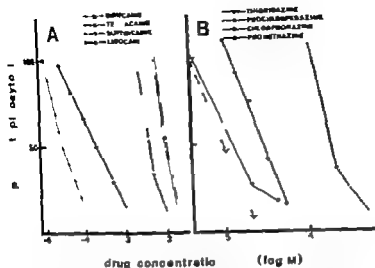


Fig. 1. Inhibition of pinocytosis after pretreatment of the cells for 25 min with local anesthetics (Fig. 1 A) benzocaine (Fig. 1 B) at pH 6.0 dissolved in 0.3 mM tris-HCl. Pinocytosis was induced by 125 mM KCl at pH 7.0. The pinocytosis intensity is given on the ordinate as percent of that induced in cells at pH 7.0 with 0.3 mM tris-HCl at no drug present. The concentrations of the drugs are given on the abscissa. Each experimental point indicates single measurement.

cently at acid and alkaline pH. This induced pinocytosis was effectively prevented when ionized drugs were applied to the cells in solutions at pH 5.5–6.5. In a series of 10 expts. tetracaine was found to be less effective at pH 7.2 than at pH 5.5 (Table I). The differences potency were greater than the differences in protonation of the drug ( $pK_a$  8.5) at these pH values. These results suggest a pH-sensitive barrier for ionized species of the drugs the cell membrane. By way of this mechanism, pH of the solution may influence the

TABLE I. Pinocytosis induced by 40 mM KCl at pH 5.7 or 100 mM tris HCl at pH 7.0 after pretreatment of the cells for 30 min with solutions of some organic bases at pH 5.5–5.7. All salts with the exception of procaine and chlorpropionate were chlorides. Controls rated at 100 are carried out on suspensions of cells taken directly from the culture fluid. Relative intensity of pinocytosis induced on suspensions of cells at pH 5.7 or 7.0 is given as the mean of 2 to 4 expts. Tris induced pinocytosis is given as percent of controls  $\pm$  S.E. of 10 expts.

Drug	pH	Conc. (M)	Inducer	Relative pinocytosis intensity
Tetracaine	5.7	$10^{-4}$	40 mM KCl	68
Procaine	5.7	$10^{-4}$	40 mM KCl	87
Chlorpropionate	5.7	$10^{-4}$	40 mM KCl	128
Procaine	5.7	$10^{-4}$	40 mM KCl	8
Procaine	5.7	$10^{-4}$	40 mM KCl	59
Procaine	5.5	$10^{-4}$	40 mM KCl	29
Procaine	5.5	$3 \cdot 10^{-4}$	40 mM KCl	34
Procaine	5.5	$3 \cdot 10^{-4}$	100 mM tris HCl	$18.3 \pm 3.1$
Procaine	7.2	$3 \cdot 10^{-4}$	100 mM tris HCl	$38.3 \pm 8.0$

For membrane potential measurement about 100 cells were transferred from the culture dish to a lucite chamber carefully washed with Pringsheim solution. The cells were allowed to adhere to the surface of the chamber and after 10 min the culture solution was substituted by the addition of 30  $\mu$ l of test solution. Conventional glass capillary microelectrodes with tip potentials not exceeding 30 mV were used for the measurement of membrane potentials. Such electrodes were obtained by selection and sometimes supported by the addition of 0.01–0.05 mM thorium nitrate to the electrolyte used for filling the electrode. For continuous longtime measurements electrodes filled with 0.3 M KCl without the addition of thorium were used. The 3 M KCl electrodes had about 20 M $\Omega$  resistance when measured in 3 M KCl. Differences in membrane potential due to thorium present in the electrode were observed when the results obtained with different types of electrodes were compared. All stimulating electrodes were filled with 3 M KCl. The measurements of membrane potential, and input resistance were made in the rear or cost part of the cell. The membrane resistance was calculated from the current necessary to be injected to make the membrane potential match a command signal applied to the cell in a voltage clamp circuit. The method has been described elsewhere (Johansson *et al.* 1975).

## Results

Preincubation of cells with local anesthetics in the culture fluid, or addition of local anesthetics of the solution of the chemical inducer did not, in general, inhibit the formation of channels. Preincubation with the same anesthetic drug concentration but in a  $\text{Ca}^{++}$ -free medium (CFP or 0.3 mM NaCl) was, however, inhibitory. At the end of a typical period of pretreatment (0.3 mM dibucaine for 30 min at pH 5.6) attachment of the cells to the glass surface was reduced and streaming of the cytoplasm was slow. The contractile vacuole was swollen and the frequency of its contractions was decreased. In high concentrations (1–25 mM) of the drugs pinocytosis cycles of low intensity were induced. The potent anesthetics dibucaine and tetracaine were less effective inducers than procaine and lidocaine. Like  $\text{Ca}^{++}$  induced pinocytosis,  $\text{Ca}^{++}$  inhibited that induced by local anesthetics.

### *Effects of pretreatment with membrane stabilizing drugs*

**Dose-response relationship.** Amoebae preincubated in solutions of local anesthetics were found to develop few pinocytotic channels in response to a strong inducer such as 100  $\mu$ M tris-HCl at pH 7.0. The magnitude of pinocytosis blockade was dependent on the concentration of the local anesthetic, the length of the incubation period and on the specific potency of the drug. Dibucaine and tetracaine were effective at low concentrations while bupivacaine (Marcaine®) and lidocaine had weaker effects (Fig. 1A). The phenothiazine drugs were equal to or more potent than the local anesthetics. The former inhibited pinocytosis induced by 100 mM tris-HCl (Fig. 1B) or 40 mM KCl (Table I) in the order: thioridazine > prochlorperazine > chlorpromazine > promethazine. Generally all these drugs inhibited potassium induced pinocytosis less efficiently than sodium or tris induced pinocytosis.

**Effect of pH.** Since the outer surface of the membrane of the amoeba is a cation exchanger (Marshall and Nachmias 1965; Hendil 1971) the membrane stabilizers could be effective as their ionized (cationic) forms. This was studied by the use of benzocaine ( $\text{pK}_a$  3.2), which exists essentially as a non-ionized free base between pH 5 and pH 8 and two quaternary local anesthetic amines (Fig. 2). In contrast to dibucaine ( $\text{pK}_a$  8.5) and the quaternary derivatives of both promethazine (N-hydroxy-ethylpromethazine) and lidocaine (QX 572) (chemical structure see Johnson and Schwartz 1969) benzocaine prevented pinocytosis

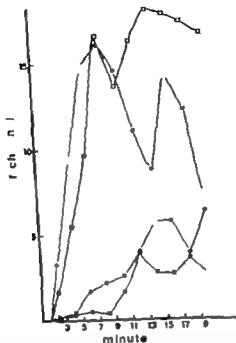


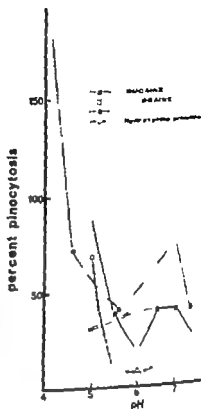
Fig. 4. Time course of channel formation during pinocytosis induced by 100 mM tris at pH 7.0. The shape of the curves varied according to the treatment of the cells in the period preceding induction of pinocytosis. Symbols indicate that cells were tested directly from the culture solution (squares) or after pretreatment with 0.3 mM NaCl for 30 min (circles). Solid circles indicate that cells were pretreated with 0.3 mM dibucaine at pH 5.6 (squares) or pH 7.0 (circles). Means of 7-10 expts. were used to make each curve.

Lower concentrations of dibucaine, required longer preincubation periods to obtain maximal effect. The period of complete blockade of pinocytosis following pretreatment was generally of short duration (Fig. 3) provided the cells were washed in calcium containing media.

**Time course of channel formation after treatment with dibucaine.** After dibucaine treatment the onset of channel formation was slow. The time dependence of channel formation following application of 100 mM tris pH 7.0 is given in Fig. 4. Cells pretreated in 0.3 mM dibucaine for 30 min at two different pH values were compared with normal cells and cells preincubated in 0.3 mM NaCl. The control cells formed maximal number of channels during 8 min and 13 min of the cycle, while channel formation induced after treatment with the local anesthetic was depressed during the first 10 min of the cycle. The partial recovery of channel formation at the end of the cycle may be analogous to recovery of pinocytosis shown in Fig. 3. In the  $\text{Ca}^{++}$ -deficient,  $\text{Na}^{+}$ -treated cells the channels appeared earlier in the pinocytosis cycle than in the control cells.

**Altered dose-response relationship for inducers after treatment with dibucaine.** Dose-response relationships for pinocytosis inducers applied to anesthetized cells were investigated to elucidate the mechanism of inhibition. Fig 5 A and B show respectively the blockade of pinocytosis induced by  $\text{K}^{+}$  and tris following pretreatment with dibucaine. With both inducers a non-competitive blockade of pinocytosis was noticed when dibucaine was applied to the

Fig. 2. Intensity of pinocytosis induced by tris (100 mM, pH 7.0) as a function of the pH (abscissa) of the drug solution after pretreatment for 30 min with four different inhibitors. The intensity of pinocytosis is given as a percentage of the control which was pretreated with 0.3 mM NaCl at the respective pH. The following concentrations and incubation periods were used: dibucaine 0.3 mM, 60 min; benzocaine 4.7 mM, 10 min; N-hydroxyethylprometazine and QX 572 0.3 mM for 30 min.



effectiveness of cations to induce pinocytosis. In all these experiments 100 mM tris pH 7.0 was the inducer; similar results were obtained with 25 mM KCl.

*Time course and reversibility of the effect of dibucaine* The effect of preincubation with local anesthetics on the subsequent blockade of tris induced pinocytosis was tested with dibucaine at pH 5.5 (Fig. 3). Preincubation for a period of at least 10 min was necessary to produce a complete blockade of pinocytosis when the dibucaine concentration was 0.3 mM.

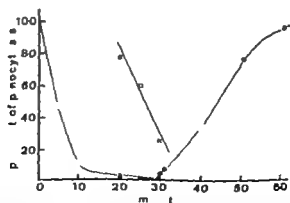


Fig. 3. Inhibition and recovery of pinocytosis induced by tris (100 mM, pH 7.0) after incubation in dibucaine 0.3 mM at pH 5.5. The intensity of pinocytosis is given on the ordinate in percentage number of channels developed in the untreated control. Solid triangles indicate pinocytosis intensity after treatment of the cells with dibucaine 0.3 mM for 5, 10, 20, 25 and 30 min. Open squares give the recovery in pinocytosis after an incubation period of 30 min in 0.3 mM dibucaine.

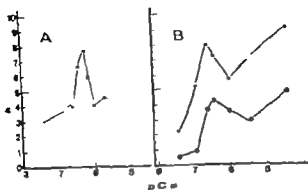


Fig. 7 A. Effect of ambient  $\text{Ca}^{++}$  concentration during potassium (25 mM pH 7.0, circles) and sodium chloride pinocytosis (100 mM pH 7.0, squares). The  $\text{Ca}^{++}$  concentrations are adjusted with an EGTA- $\text{Ca}^{++}$  buffer. The calculated  $\text{Ca}^{++}$  concentration is given as pCa on the abscissa.

Fig. 7 B. Pinocytosis induced by KCl (20 mM pH 7.0) in cells pretreated for 30 min at pH 5.6 in 0.3 mM NaCl (solid circles) or 0.3 mM dibucaine (open circles). In addition to inhibition at all  $\text{Ca}^{++}$  concentrations the latter curve displayed a shift to the right from the control curve.

Effect on pinocytosis (induced by tris or  $\text{K}^+$ ), sodium thiopental (1 mM), and tetrodotoxin (3.61  $\mu\text{g}/\text{ml}$ ) were found to be without effect when applied 30 min before, or during pinocytosis. The organic bases, imipramine and desmethylinipramine, however, possessed an inhibitory effect comparable in strength to that of dibucaine. Likewise vinblastine sulphate (0.3–0.5 mg/ml pH 5.6) applied to the cells in CFP 30 min before induction inhibited tris induced pinocytosis by 95 percent (Fig. 6 A) and  $\text{K}^+$ -induced pinocytosis by 75 percent. Recovery from inhibition occurred when the cells were transferred to Pringsheim solution (Fig. 6 B). Colchicine (up to 10 mM) slightly stimulated tris induced pinocytosis.

**Effects of dibucaine pretreatment on pinocytosis in the presence of  $\text{Ca}^{++}$**  As described previously (Jordeson 1968), pinocytosis induced by low concentrations of monovalent cations is stimulated by small amounts of the  $\text{Ca}^{++}$  chelating agent EGTA, while a low pCa tends to block pinocytosis. It seemed that, by the use of a  $\text{Ca}^{++}$  buffer system it should be possible to find optimal  $\text{Ca}^{++}$  levels for different types of pinocytosis. Fig. 7 A illustrates such experiments for KCl and NaCl induced pinocytosis. When the  $\text{Ca}^{++}$  concentration of the inducing KCl solution (40 mM) was varied by the use of 5 mM Ca-EGTA buffer optimal pinocytosis was found at a calculated  $\text{Ca}^{++}$  concentration of about  $10^{-4.5}$  M. In similar experiments (not shown) the shape of the pCa-pinocytosis curve was the same in both 10 and 20 mM KCl. With 100 mM KCl a second optimal  $\text{Ca}^{++}$  concentration at about pCa 5 was noted. The pinocytosis induced by NaCl (100 mM), appeared to be maximally stimulated at a still higher  $\text{Ca}^{++}$  concentration (Fig. 7 A).

Such experiments were also carried out on cells pretreated with 0.3 mM NaCl or dibucaine-HCl. It was found that the curve after dibucaine-HCl treatment (Fig. 7 B) describing potassium induced pinocytosis as a function of pCa was shifted towards higher  $\text{Ca}^{++}$  concentrations but retained the shape typical for cells pretreated with NaCl. These experiments might indicate that dibucaine depletes the cells of  $\text{Ca}^{++}$ . By measuring the  $\text{Ca}^{++}$  content of the extracellular medium (Fig. 8) it was confirmed, that dibucaine released more  $\text{Ca}^{++}$  from the

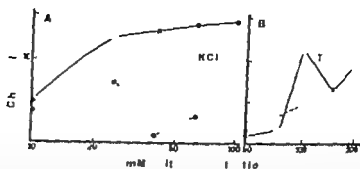


Fig. 5 A. Dose-response relationship for potassium induced pinocytosis in cells pretreated for 30 min with 0.3 mM NaCl pH 5.8 (solid line) or 0.3 mM dibucaine pH 5.6 (broken line).

Fig. 5 B. Dose-response relationship for tris induced pinocytosis in cells pretreated for 30 min with 0.3 mM NaCl (solid line) or in dibucaine 0.1 mM pH 5.6 (broken line).

cells 30 min before the experiment. In Fig. 5 A the intensity of pinocytosis induced by 100 mM KCl is given for cells pretreated with 0.3 mM of dibucaine or as a control with NaCl. Treatment with the latter solution did not prevent a normal pinocytosis cycle but appeared to abolish the "auto-inhibition" of high  $K^+$ -concentrations, as described earlier (Josefsson 1968). Treatment with dibucaine prevented channel formation in all KCl solutions and especially at 40 mM KCl where pinocytosis in normal cells is most intense. Compared to controls in 0.1 mM NaCl, dibucaine did not prevent pinocytosis in response to low concentrations of tris (Fig. 5 B). The pinocytosis induced by 100 mM and higher concentrations of tris was, however, markedly depressed.

*Effects of some other drugs on the capacity for pinocytosis* In a survey for drugs which

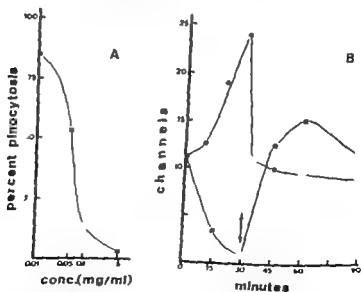
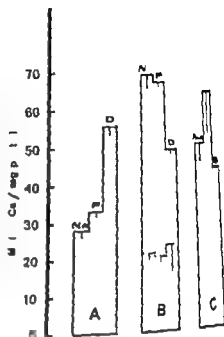


Fig. 6 A. Effect of pretreatment for 30 min with vincristine sulphate on pinocytosis induced by 100 mM tris pH 7.0. The concentration of the drug, dissolved in CFP at pH 5.6, is given on the abscissa. The ordinate indicates the intensity of pinocytosis as percent of the control cells treated in CFP.

Fig. 6 B. Time course of inhibition of tris-induced pinocytosis by 0.5 mg/ml vincristine sulphate in CFP at pH 5.6 (squares). For comparison the pinocytosis intensity after treatment with CFP but without the drug is indicated by circles in the figure. Note that the intensity of pinocytosis decreases in the course of treatment with the CFP solution. After 30 min of treatment (arrow) the cells were washed and transferred to Pringle's solution. With both types of pretreatment normal pinocytosis was restored within 15 min.



Fig. 8. Calcium in cells and media after incubation at pH 5.6 in 0.3 mM of NaCl (Na), procaine (P) or dibucaine (D) as analyzed with absorption spectrophotometry. The calcium values are related to the protein content (Lowry 1951) of the cell pellet. Column A gives the amount of calcium which was released to the extracellular medium after 30 min incubation in these solutions. All cells were washed in 1 mM NaCl pH 6.0 thereafter incubated in 1 mM  $\text{LaCl}_3$  at pH 5.6 for another 30 min. Column B gives the amount of calcium removed from the cells during Na (dotted line) and  $\text{La}^{+++}$  (solid lines) treatment. After a final wash in NaCl (1 mM) the calcium which remained in the cell pellet after  $\text{La}^{+++}$  treatment is shown in column C. Each column in A represent 8 and in B and C 4 individual expts. S.E. are given as vertical bars in the columns.



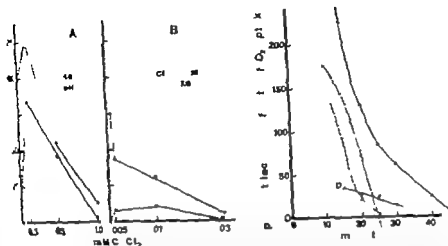
cells than did NaCl or procaine (Fig. 8 column A). Of the total cell calcium the  $\text{La}^{+++}$  sensitive portion which probably corresponds to  $\text{Ca}^{++}$  bound to the cell surface, was low in dibucaine treated cells (Fig. 8 column B). The calcium which remained was however not significantly lowered (column C) after the cells had been incubated in dibucaine or procaine solution followed by  $\text{LaCl}_3$ . The loss of  $\text{Ca}^{++}$  from the cells could, however not be directly responsible for the inhibitory effect of dibucaine since comparable or greater  $\text{Ca}^{++}$ -release occurred in media containing EGTA and these failed to inhibit pinocytosis (Table II).

In previous studies it has been shown that  $\text{Ca}^{++}$  inhibits channel formation when present in the period preceding induction or during the pinocytosis cycle (Brandt and Freeman 1962, Cooper 1968, Josefsson 1968). Since local anesthetics might inhibit pinocytosis by a  $\text{Ca}^{++}$

TABLE II Comparison between  $\text{Ca}^{++}$  release from the cell and pinocytosis intensity. Amoebic cultures in Pringsheim solution were incubated for 30 min in different media (left column). All solutions were at pH 5.6 except the sodium salt of EGTA which was at pH 7.0. The release of calcium into the medium was measured by absorption spectrophotometry and the values are given as percent of the total calcium content of the sample  $\pm$  S.E. After incubation in respective solutions, aliquots of the cells were tested for capacity to form channels in 100 mM tris-HCl at pH 7.0.

Extracellular medium	$\text{Ca}^{++}$ -release/30 min (% of total Ca)	Pinocytotic response to inductor (% of max)
Distilled water	20 $\pm$ 0.4 (4)	33
Pringsheim	—	57
N Cl 0.3 mM	23 $\pm$ 2.3 (3)	100
$\text{Na}_2\text{EGTA}$ 0.05 mM	36 $\pm$ 4.7 (4)	87
CFP	26 $\pm$ 1.3 (8)	61
$\text{Na}_2\text{EGTA}$ 0.1 mM	57 $\pm$ 1.6 (4)	31
Dibucaine 0.1 mM	30 $\pm$ 3.8 (4)	15
$\text{Na}_2\text{EGTA}$ 0.3 mM	64 $\pm$ 1.2 (4)	8
Dibucaine 0.3 mM	31 $\pm$ 2.0 (12)	1





9. Effect of dibucaine pretreatment on calcium inhibition of potassium (A) and sodium induced pinocytosis (B). The  $\text{Ca}^{++}$  concentrations of the inducing solution is given on the abscissa. The pinocytosis rate given on the ordinates is percentage of the pinocytosis elicited by the inducers 40 mM KCl pH 7.0 and 100 mM NaCl pH 7.0. Each experimental point indicates single measurement on normal cells (control) and cells pretreated for 30 min with 0.3 mM dibucaine pH 5.6 (squares).

10. Rate of oxygen uptake after induction of pinocytosis (curves A, B and D) and after the exchange of one sodium (curve C). The measurements were made with a constant micro-driver at 25°C. Pinocytosis induced by 100 mM Tris-HCl pH 7.0. Oxygen uptake is given as percentage increase from the steady rate of  $\text{O}_2$  uptake measured for 10 min period before the introduction of the inducing solution at time. All curves start at ordinate zero. The manually controlled driver technique did not allow measurements to be made earlier than 10 min after the exchange of solution. Curve C indicates the normal transport rates as measured following exchange (at zero time) of *Pyraghiana* solution in the micro-driver. Curve B indicates the respiratory increase in control cells during pinocytosis which follows upon exchange of 0.3 mM KCl for the inducing solution. Curve A represents conditions as for curve B with the exception that 0.3 mM dibucaine is present in the inducing solution. Curve D gives the increase of  $\text{O}_2$  uptake during pinocytosis in cells pretreated for 30 min with 0.3 mM dibucaine. The conditions in A and D correspond to stimulated and inhibited pinocytosis respectively. Each experimental point is the mean of 5 experiments.

splitting action, the interaction between the two types of pinocytosis blockers was examined. The interference of dibucaine with  $\text{Ca}^{++}$  inhibited pinocytosis was investigated in 3 mM KCl (pH 5.7) or 100 mM NaCl (pH 7.0) (Fig. 9 A and B). No additive effects of the two types of inhibitors were observed. On the contrary dibucaine pretreatment partly counteracted the  $\text{Ca}^{++}$  inhibition of pinocytosis. The intensity of pinocytosis induced by 40 mM KCl in the presence of 0.5–1 mM  $\text{CaCl}_2$  did not differ significantly in control cells and cells pretreated with dibucaine. Using NaCl (100 mM, pH 5.7 and 7.0) or Tris HCl (100 mM pH 7.0) as inducing salts prevention of pinocytosis by dibucaine was, however, only partially counteracted when  $\text{Ca}^{++}$  was added to the inducer.

#### *Oxygen uptake after dibucaine treatment*

During pinocytosis induced by several monovalent inorganic cations the oxygen uptake of the amoeba was increased (Hansson *et al.* 1968). This metabolic response to the pinocytosis inducer was reduced when  $\text{Ca}^{++}$  was added in concentrations which prevented pinocytosis. It was also found that mechanical manipulations of the cells as well as factors which stimulate channel formation increased the rate of respiration. The latter was measured with the

TABLE III Relative rate of oxygen in Pringsheim solution measured with a Clark electrode in SCT cells were pre-focubated at room temperature with respective drug for 30 min. Respiration taken within 5 to 10 min after the cells were transferred into Pringsheim. The figures are percent of the rates  $\pm$  S.E. recorded in Pringsheim after incubation of aliquots of cells (about 50) in 0.3 mM NaCl. The number of experiments is given within brackets.

Pretreatment	Relative rate of $O_2$ -uptake $\pm$ S.E.
Dibucaine 0.3 mM pH 5.6	69 $\pm$ 6.6 (9)
Bupivacaine 0.3 mM pH 5.6	78 $\pm$ 5.7 (7)
Procaine 0.3 mM pH 5.6	88 $\pm$ 3.6 (7)
Vinblastine 0.3 mM pH 5.6	71 $\pm$ 6.5 (7)
NaCl 0.3 mM pH 5.8	100 (control)

cartesian diver technique during pinocytosis in 100 mM tris HCl pH 7.0. The results given in Fig. 10 where curve B represents the relative oxygen uptake during a normal cycle of pinocytosis. It is evident from the figure that the presence of dibucaine, which inhibits pinocytosis increased the rate of respiration (curve A), while pretreatment with the drug strongly depressed oxygen uptake (curve D). These findings with dibucaine are consistent with the view (Hansson *et al.* 1968) that the degree of respiration is closely connected with channel formation. Pretreatment with the drug seemed to erase not only the effect of inducer on respiration but also the normal respiratory increase after exchange of extracellular medium (curve C). From previous experiments with EGTA the rate of oxygen consumption was suggested to be determined by the  $Ca^{++}$  concentration in the cell membrane. Stimulated respiration was observed when the  $Ca^{++}$ -level was lowered from that prevailing in the culture condition, while respiration was slowed down in cells both in  $Ca^{++}$  rich and in  $Ca^{++}$  deficient media. The different rates of respiration observed in the presence of dibucaine after pretreatment with the drug might therefore reflect different degrees of  $Ca^{++}$  deficiency.

Measurements with a Clark electrode on suspensions of cells revealed that respiration of the cells in the culture medium was depressed after pretreatment with the drugs (Table I). The difference between the potent inhibitors of pinocytosis (dibucaine, vinblastine) and indifferent or stimulating drugs (bupivacaine, procaine) was however small, so that interference with oxygen metabolism is not likely to determine the actions of these drugs on pinocytosis.

#### *Effects of dibucaine on membrane potential and input resistance*

When cells were transferred from culture fluid to a solution of 0.3 mM dibucaine, marginal changes of the membrane potential occurred during the subsequent 20 min (Fig. 11 A). As reported previously (Josefsson *et al.* 1975) a prompt depolarization of amoeba occurred when simple monovalent cations were added to the cells in concentrations which induce channel formation. Pretreatment with dibucaine, which completely prevents induced pinocytosis, did not, however prevent depolarization due to the inducing cations (Table IV). Dibucaine also failed to reduce the increase in membrane conductance which followed the addition of these cations. This is illustrated in Fig. 11 B and C. In Fig. 11 B the input resistance of a cell was repeatedly measured during the pinocytosis period (in 20 mM NaCl) both in the culture fluid and during incubation with dibucaine. The de-

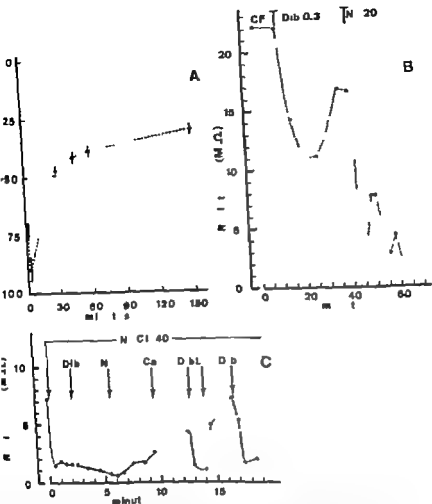


Fig. 11 A Membrane potentials measured in cells incubated in 0.3 mM dibucaine pH 5.6 for varying lengths of time (abscissa). The values at zero time were obtained in Pringle's solution. Experimental points indicate the mean potential values  $\pm$  S.E. obtained from 8–12 cells.

B Input resistance of cell in culture fluid (CF) followed by treatment with 0.3 mM dibucaine pH 5.6 and followed 30 min later by treatment with the inducer 20 mM NaCl pH 5.9. The ordinate gives resistance also expressed in MΩ obtained by the voltage clamp technique. The experiment simulates the procedure used for the study of pinocytosis inhibition by drugs.

C Effect of dibucaine  $\text{Ca}^{++}$  and  $\text{La}^{+++}$  on input resistance of cell suspended in 40 mM NaCl. In the course of the experiment the medium was exchanged (arrow in the figure) without altering the pH and NaCl concentration. The media used were introduced in the following order: 0.2 mM dibucaine, wash in 40 mM NaCl, 0.3 mM  $\text{CaCl}_2$ , 0.2 mM dibucaine, 0.5 mM  $\text{LaCl}_3$ , 0.2 mM dibucaine. Note that the presence of dibucaine coincides with low input resistance.

of membrane resistance upon incubation with the inducer was as prompt and as profound as in normal cells. In Table V the input resistance of normal and of dibucaine treated cells in three different solutions is summarized. In these media the anesthetized cells had the lower resistance. Fig. 11 C demonstrates that the drug added to the inducer decreased the input

TABLE III Relative rate of oxygen in Pringsheim solution measured with a Clark electrode at 30°C. Cells were pre-incubated at room temperature with respective drug for 30 min. Readings taken within 5 to 10 min after the cells were transferred to Pringsheim. The figures are percentages of the rates  $\pm$  S.E. recorded in Pringsheim after incubation of aliquots of cells (about  $1 \times 10^6$ ) in 0.3 mM NaCl. The number of experiments is given within brackets.

Pretreatment	Relative rate of $O_2$ -uptake $\pm$ S.E.
Dibucaine 0.3 mM pH 5.6	69 $\pm$ 6.6 (9)
Bupivacaine 0.3 mM pH 5.6	78 $\pm$ 5.7 (7)
Procaine 0.3 mM pH 5.6	88 $\pm$ 3.6 (7)
Vinblastine 0.3 mM pH 5.6	71 $\pm$ 6.5 (7)
NaCl 0.3 mM pH 5.8	100 (control)

cartesian diver technique during pinocytosis in 100 mM tris HCl pH 7.0. The result given in Fig. 10 where curve B represents the relative oxygen uptake during a normal pinocytosis. It is evident from the figure that the presence of dibucaine, which stimulates pinocytosis increased the rate of respiration (curve A), while pretreatment with the drug strongly depressed oxygen uptake (curve D). These findings with dibucaine are consistent with the view (Hansson *et al.* 1968) that the degree of respiration is closely connected with channel formation. Pretreatment with the drug seemed to erase not only the effect of the inducer on respiration but also the normal respiratory increase after exchange of culture medium (curve C). From previous experiments with EGTA the rate of oxygen consumption was suggested to be determined by the  $Ca^{++}$  concentration in the cell membrane. Stimulated respiration was observed when the  $Ca^{++}$  level was lowered from that prevailing in the culture condition, while respiration was slowed down in cells both in  $Ca^{++}$  rich and in  $Ca^{++}$  deficient media. The different rates of respiration observed in the presence of dibucaine after pretreatment with the drug might therefore reflect different degrees of  $Ca^{++}$  deficiency.

Measurements with a Clark electrode on suspensions of cells revealed that respiration of the cells in the culture medium was depressed after pretreatment with the drugs (Table III). The difference between the potent inhibitors of pinocytosis (dibucaine, vinblastine) and the indifferent or stimulating drugs (bupivacaine, procaine) was however small, so the interference with oxygen metabolism is not likely to determine the actions of these drugs on pinocytosis.

#### *Effects of dibucaine on membrane potential and input resistance*

When cells were transferred from culture fluid to a solution of 0.3 mM dibucaine, marginal changes of the membrane potential occurred during the subsequent 20 min (Fig. 11 A). As reported previously (Josefsson *et al.* 1975) a prompt depolarization of the amoeba occurred when simple monovalent cations were added to the cells in cocoon solution which induce channel formation. Pretreatment with dibucaine, which completely prevents induced pinocytosis, did not, however, prevent depolarization due to the induction (Table IV). Dibucaine also failed to reduce the increase in membrane conductance which followed the addition of these cations. This is illustrated in Fig. 11 B and C. In Fig. 11 D the input resistance of a cell was repeatedly measured during the pinocytosis period (in 20 mM NaCl) both in the culture fluid and during incubation with dibucaine. The

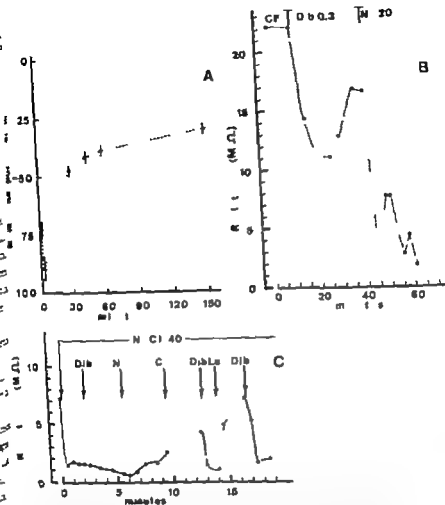


Fig. 11 A. Membrane potentials measured in cells incubated in 0.3 mM dibucaine pH 5.6 for varying lengths of time (abscissa). The values at zero time were obtained in Fruingera solution. Experimental points indicate the mean potential value. S.E. obtained from 8-12 cells.

B. Input resistance of cell in culture fluid (CF) followed by treatment with 0.3 mM dibucaine pH 5.6 followed 30 min later by treatment with the inducer 20 mM NaCl pH 5.9. The ordinate gives resistance also expressed as MΩ obtained by the voltage clamp technique. The experimental replicates the procedure used for the study of penocytosis inhibition by drugs.

C. Effect of dibucaine  $\text{Ca}^{++}$  and  $\text{La}^{+++}$  on input resistance of cell immersed in 40 mM NaCl. In the course of the experiment the medium was exchanged (arrows in the figure) without altering the pH and NaCl concentration. The media used were introduced in the following order: 0.2 mM dibucaine, wash in 40 mM NaCl, 0.5 mM  $\text{CaCl}_2$ , 0.2 mM dibucaine, 0.5 mM  $\text{LaCl}_3$ , 0.4 mM dibucaine. Note that the presence of dibucaine coincides with low input resistance.

of membrane resistance upon incubation with the inducer was as prompt and as profound as in normal cells. In Table V the input resistance of normal and of dibucaine treated cells in three different solutions is summarized. In these media the anesthetized cells had the lower resistances. Fig. 11 C demonstrates that the drug added to the inducer decreased the input

TABLE IV The effect of dibucaine on membrane potential in *Amoeba proteus*. The cells were for 30 min either in Pringsheim pH 6.8 or 0.3 mM NaCl pH 5.8 or 0.3 mM  $\Delta$ NaCl. Recording was made during a 10 min period following the incubation of the cells in test solution. Tris 100 mM at pH 7.0 was used as pinocytosis inducer. Membrane potential in mV  $\pm$  S.E. 13-20 measurements were made per experiment.

Pretreatment	Test solution	mV
Pringsheim	Dibucaine	$-90.3 \pm 6.1$
Pringsheim	NaCl	$-85.5 \pm 6.4$
Pringsheim	Tris 100 mM	$-12.0 \pm 1.0$
Dibucaine	Tris 100 mM	$-19.6 \pm 1.1$
NaCl	Tris 100 mM	$-17.7 \pm 0.6$

resistance even when the cells had been pretreated with  $\text{Ca}^{++}$  or  $\text{La}^{+++}$  contain. It may be concluded that in contrast to  $\text{Ca}^{++}$  + dibucaine prevents pinocytosis increasing either the input resistance or the membrane potential of the cell.

### Discussion

For a local anesthetic to prevent channel formation it was necessary that the contact with the cell, in a medium of low  $\text{Ca}^{++}$  concentration for a certain period before addition of the inducing monovalent cation. This requirement and the fact that at about pH 6 for the inhibitory effect might indicate that the drugs were taken up by pinocytosis. Like  $\text{Ca}^{++}$  these drugs were weak inducers of pinocytosis in the amoeba, unlike  $\text{Ca}^{++}$  they had no stabilizing effect on the electrical properties of the cell membrane, but the depression of pinocytosis could be correlated to the depression of pinocytosis. Yet the order of potency of the drugs to inhibit pinocytosis was similar to the order of their anesthetic potency (Aronow 1963, Truant and Takman 1965), and to the order in which the drugs inhibit calcium to phospholipids (Blaustein and Goldman 1966 b). Furthermore the effect of pH and local anesthetics on pinocytosis was similar to the sequences given by Weinstein (1966) and Seeman (1966) for stabilizing erythrocytes against osmotic lysis. This indicates that prevention of pinocytosis is another reflection of the membrane effect of these drugs. Local anesthetic decreased respiration in the amoeba and dissociation of the bioelectrical effects of the inducer from the morphological and electrical phenomena of pinocytosis. This is in accord with their inhibition of contractility caused by depolarization in conditions of excess extracellular  $\text{K}^{+}$  viz. contracture (Novotny *et al.* 1962), respiratory increase in muscle (Novotny and Vyskocil 1962).

TABLE V The effect of dibucaine pretreatment (0.3 mM  $\pm$  10 min) on input resistance.  $M\Omega \pm$  S.E. The number of cells are in this brackets. The pH of the test solutions.

Test solution	Control cells	Dibucaine treated cells
NaCl 40 mM	$9 \pm 0.21$ (48)	$4 \pm 0.25$ (4)
KCl 40 mM	$0.7 \pm 0.17$ (8)	$0.4 \pm 0.11$ (6)
KCl 10 mM	$2.7 \pm 0.53$ (7)	$2 \pm 0.16$ (10)

ated secretion in the adrenal medulla (Jaanus *et al.* 1967). In these tissues  $\text{Ca}^{++}$  ex-  
e is also decreased by local anesthetic drugs (Littigan and Ootliker 1968, Rubin *et al.*  
Thus when the coupling between excitation and contraction or between stimulus  
ejection are interrupted by local anesthetic drugs the binding and the movement of  
in membrane structures seems to be inhibited. It appears that a similar mechanism of  
might be relevant for the prevention of pinocytosis as reported here.  
e release of surface  $\text{Ca}^{++}$  from the amoebae in dibucaine solutions, the augmented  
of the drug in the presence of calcium binding agents and the weak inhibitory effect  
e drug on  $\text{Ca}^{++}$ -depressed pinocytosis, points towards inhibition of pinocytosis due to  
deficiency. Surface  $\text{Ca}^{++}$  controls membrane permeability to ions (Brandt and Freeman  
Brandt and Hendil 1970, 1972, Josefson *et al.* 1975). Drugs which release  $\text{Ca}^{++}$  would  
fore, as observed here with dibucaine, decrease transmembrane resistance and potential.  
strong effect of the drugs on sodium and tris induced pinocytosis compared to pino-  
is induced by K<sup>+</sup> might indicate, that surface calcium plays a more dominant role in  
cytosis induced by Na<sup>+</sup> or tris ions. This is further supported by the observation that the  
ence of EGTA depresses tris (Josefson 1968) and sodium induced pinocytosis more than  
sodium induced pinocytosis (see Fig. 7). Interference with the calcium metabolism in the  
cells might also be responsible for the depressed oxygen uptake, especially subsequent to  
application of the inductor since high concentrations of EGTA have been shown to  
ruberly decrease respiration in this cell (Hansson *et al.* 1968). The mere loss of  $\text{Ca}^{++}$  from  
cell surface in the presence of dibucaine could, however not explain the decreased channel  
eformation. The drug might, in addition to its action as a simple counterion to fixed anions  
be surface membrane, penetrate into the lipids of the plasma membrane and displace  
from polar groups in this structure and even possibly from intracellular membranes.  
e local anesthetics release membrane calcium from artificial lipid membranes and from  
ve and muscle (Hauser and Dawson 1968, Kuperman *et al.* 1968), and the cationic form  
dibucaine, when bound to the surface of the nerve membrane reduces the uptake of  
eocalcium (Suzer-Kurtz *et al.* 1970). Chlorpromazine and Imipramine, both potent  
e inhibitors of pinocytosis, have also been shown to inhibit calcium transport in biological  
e membranes (Balzer *et al.* 1968). It might be that  $\text{Ca}^{++}$  bound in the plasma membrane of the  
e cells has to be released into the cytosol for the onset of channel formation. Replacement  
the Ca<sup>++</sup> fraction by lipophilic organic bases in general might therefore directly inhibit  
e factors which exchange with calcium at plasma membrane or otherwise cause calcium  
e case form this site.  
The effects of vinblastine on the amoeba were very similar to those of dibucaine. This drug  
e is no anesthetic action but like other lipid soluble cationic drugs it expands membranes  
e and protects erythrocytes from osmotic hemolysis (Seeman *et al.* 1973). Like colchicine,  
e which did not actually inhibit pinocytosis, vinblastine is known to affect microtubules (Mar-  
e tz *et al.* 1969). It should be emphasized that only high concentrations of vinblastine in-  
e hibited pinocytosis in the amoeba and that unspecific effects like those described by Seeman  
e *et al.* (1973), common for lipid soluble organic bases, might therefore dominate. Recently  
e Sloan *et al.* (1970) found that vinblastine sulphate could precipitate a number of proteins  
e derived from cell structures all of which were also precipitated by  $\text{Ca}^{++}$ . They concluded that

TABLE IV The effect of dibucaine on membrane potential in *Ameba proteus*. The cells were preincubated for 30 min either in Pringsheim pH 6.8 or 0.3 mM NaCl pH 5.8 or 0.3 mM dibucaine pH 5.8. Recording was made during a 20 min period following the incubation of the cells in a test solution. Tris 100 mM at pH 7.0 was used as pinocytosis inducer. Membrane potential in mV  $\pm$  S.E. 15–20 measurements were made per experiment.

Pretreatment	Test solution	mV
Pringsheim	Dibucaine	$-90.3 \pm 6.1$
Pringsheim	NaCl	$-85.5 \pm 6.4$
Pringsheim	Tris 100 mM	$-22.0 \pm 1.0$
Dibucaine	Tris 100 mM	$-19.6 \pm 1.1$
NaCl	Tris 100 mM	$-17.7 \pm 0.6$

resistance even when the cells had been pretreated with  $\text{Ca}^{++}$  or  $\text{La}^{+++}$  containing solutions. It may be concluded that in contrast to  $\text{Ca}^{++}$  dibucaine prevents pinocytosis without increasing either the input resistance or the membrane potential of the cell.

### Discussion

For a local anesthetic to prevent channel formation it was necessary that the drug be in contact with the cell in a medium of low  $\text{Ca}^{++}$  concentration for a certain period of time before addition of the inducing monovalent cation. This requirement and the pH optimum at about pH 6 for the inhibitory effect might indicate that the drugs were taken into the cell by pinocytosis. Like  $\text{Ca}^{++}$  these drugs were weak inducers of pinocytosis in the amoeba, unlike  $\text{Ca}^{++}$  they had no stabilizing effect on the electrical properties of the cell which could be correlated to the depression of pinocytosis. Yet the order of potency of the drugs in inhibiting pinocytosis was similar to the order of their anesthetic potency (Acres and Ma 1963, Truant and Takman 1965) and to the order in which the drugs inhibit calcium binding to phospholipids (Blaustein and Goldman 1966 b). Furthermore the effect of phenothiazines and local anesthetics on pinocytosis was similar to the sequences given by Seeman and Weinstein (1966) and Seeman (1966) for stabilizing erythrocytes against osmotic hemolysis. This indicates that prevention of pinocytosis is another reflection of the membrane stabilizing effect of these drugs. Local anesthetics decreased respiration in the amoeba and so caused dissociation of the bioelectrical effects of the inducer from the morphological and physiological phenomena of pinocytosis. This is in accord with their inhibition of phenocytosis caused by depolarization in conditions of excess extracellular  $\text{K}^{+}$  viz: contractures in muscle (Novotný *et al.* 1962), respiratory increase in muscle (Novotný and Vykočil 1966) and (

TABLE V The effect of dibucaine pretreatment (0.3 mM for 30 min) on input resistance. The values are  $\text{M}\Omega \pm \text{S.E.}$  The number of cells are within brackets. The pH of the test solutions was 5.8.

Test solution	Control cells	Dibucaine treated cells
NaCl 40 mM	$2.9 \pm 0.21$ (48)	$2.4 \pm 0.3$ (24)
KCl 40 mM	$0.7 \pm 0.17$ (8)	$0.4 \pm 0.11$ (6)
LiCl 10 mM	$2.7 \pm 0.35$ (7)	$2.2 \pm 0.18$ (10)



4. O. M., M. J. ROSENBOUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin reagent. *J. Biol. Chem.* 1951 193, 265-275.
5. ALI, H. C. and H. OETIKER, The action of caffeine on the activation of the contractile mechanism of muscle fibers. *J. Physiol. (Lond.)* 1968 194 51-74.
6. VIT, R., M. VENTILLA and M. SZILANSKI, Vinblastine-induced precipitation of microtubule proteins. *Proc. Natl. Acad. Sci. (U.S.A.)* 1969 66 498-499.
7. MILL, J. M. and V. T. NACHREIN, Cell surface and pinocytosis. *J. Histochem. Cytochem.* 1965 13, 104.
8. VIT, I. and F. VYKOCIL, Possible role of Ca ions in the resting metabolism of frog sartorius muscle during potassium depolarization. *J. cell comp. Physiol.* 1966 67 159-168.
9. VIT, I., F. VYKOCIL, L. VYKOCILY and R. BILANSKY, Potassium and caffeine induced increases of gas consumption in frog muscle and its inhibition by drugs. *Physiol. Bohemoslov.* 1962 11 277-284.
10. R. P. M. B. FERREIRA, S. D. JAMES and M. PARKER, Inhibition of catecholamine secretion and ion exchange in perfused rat adrenal glands by tetraacetic acid and ouabain. *J. Pharmacol. exp. Ther.* 1971 213 463-471.
11. ALLENBACH, G. Die komplexometrische Titration. 4. Auflage, Stuttgart 1960.
12. A. P. Erythrocyte membrane stabilization by local anesthetics and tranquilizers. *Biochem. Pharmacol.* 6 15, 1753-1766.
13. A. P. III CHAN-WONG and S. MOYED, Membrane expansion by vinblastine and strychnine. *Nature Biol.* 1973 241 22.
14. A. P. and I. WERTS, Erythrocyte membrane stabilization by tranquilizers and antihistamines. *chem. Pharmacol.* 1966 15 1737-1752.
15. K-KRITZ, G., C. P. BLANCHI and P. KRAUPP, Effect of local anesthetics on radioisotope binding in rat brain. *J. Pharmacol.* 1970 70, 91-100.
16. A. P. and B. TADMAN, Local Anesthetics. In *Dress' Pharmacology in Medicine*, 3rd Edition, ed. by D. Palmer, New York, McGraw-Hill, 1965, 143-156.
17. OL, L., J. BEVAN, A. RUBY and D. MARIA, Precipitation of proteins by vinblastine and calcium ions. *Proc. Natl. Acad. Sci. (U.S.A.)* 1970 66, 807-814.

vinblastine competed with  $\text{Ca}^{++}$  for binding sites on these proteins providing support for the hypothesis that interference with  $\text{Ca}^{++}$  binding is also the basis for the inhibition of pinocytosis by this drug. On the present evidence concerning  $\text{Ca}^{++}$  and pinocytosis, experiments indicate that organic bases act through an inhibitory effect on a  $\text{Ca}^{++}$  channel which couples the absorption of the chemical inducer in the membrane to the closure of channels.

This work was supported by the Swedish Cancer Society project number 167 k.69-02X and the Faculty of the University of Lund, Sweden.

The following firms graciously donated drug samples: AB Leo, Helsingborg, Sweden (thioridazine HCl and prochlorperazine methanesulphonate), AB Astra, Södertälje, Sweden (lidocaine-HCl no. 572), AB Bofors, Mölndal, Sweden (mephivacaine-HCl, bupivacaine (Marcain®)), E. Lilly, Indianapolis, USA (vinblastine sulphate), Sandoz, Basle, Switzerland (thioridazine-HCl), AB Recepta, Lund, Sweden (promethazine-HCl and N-hydroxyethylpromethazine HCl (Aprobi®)), Ciba, Basle, Switzerland (dibucaine).

## References

- ACERVO, J. and A. MACHINEZ, The action of calcium and of local anesthetics on nerve cells, and their action during excitation. *J. Pharmacol. exp. Ther.* 1963, **140**, 134-148.
- BAKKE, H. and M. MARMOSE, The inhibition of the sarcoplasmic calcium pump produced by: which react with its lipid component reserpine, procylamine, chlorpromazine, imipramine. *A. Schmiedeberg Arch. exp. Path. Pharmacol.* 1968, **259**, 151.
- BLAUSTEIN, M. P. and D. E. GOLDMAN, Competitive action of calcium and procaine on  $\text{K}^{+}$  channels. *J. gen. Physiol.* 1966, **49**, 1047-1063.
- BLAUSTEIN, M. P. and D. E. GOLDMAN, Action of anionic and cationic nerve-blocking agents. *Exp. and Interpretation. Science* 1966, **153**, 429-432.
- BRANDT, P. W. and A. R. FREEMAN, Plasma membrane Substructural changes correlated with cell resistance and pinocytosis. *Science* 1967, **155**, 382-383.
- BRANDT, P. W. and K. B. HENDIL, Properties of the plasma membrane of amoeba. In: Blah, M. *Advance in Exp. Medicine and Biology* 1970, **7**, 323-335.
- BRANDT, P. W. and K. B. HENDIL, Plasma membrane permeability and pinocytosis in *Chlamydomonas*. *Lab. Carlsberg* 1972, **38**, 423-443.
- COOPER, B. A., Quantitative studies of pinocytosis induced in *Amoeba proteus* by sample columns. *Lab. Carlsberg* 1968, **36**, 385-403.
- FENKEL, M. B., Reaction of local anesthetics with phospholipids. A possible chemical basis for action. *J. gen. Physiol.* 1964, **48**, 357-374.
- HANSSON, S. E., G. JOHANSSON and J.-O. JOSEFSSON, Oxygen uptake during induced pinocytosis in *A. proteus*. *Acta physiol. scand.* 1968, **73**, 491-500.
- HAUSER, H. and R. M. C. D. WILSON, The displacement of calcium ions from phospholipid membranes by pharmacologically active and other organic bases. *Biochem. J.* 1968, **109**, 909-916.
- HENDIL, K. B., Ion exchange properties of the glycocalyx of the amoeba *Chlamydomonas* and its role in pinocytosis. *C. R. Lab. Carlsberg* 1971, **38**, 187-211.
- JAAHAN, S. D. E. MILLER and R. P. RUBIN, The analysis of the inhibitory effect of local anesthetics propofol on adrenomedullary secretion evoked by calcium or acetylcholine. *Br. J. Pharmacol.* 1967, **31**, 319-330.
- JONSSON, C. L. and A. SCHWARTZ, Some effects of local anesthetics on isolated mitochondria. *J. Pharmacol. exp. Ther.* 1969, **67**, 365-373.
- JOSEFSSON, J.-O., Induction and inhibition of pinocytosis in *Amoeba proteus*. *Acta physiol. scand.* 1973, **73**, 481-490.
- JOSEFSSON, J.-O., N.-G. HOLMER and S. E. HANSSON, Membrane potential and conductance during pinocytosis induced in *Amoeba proteus* with alkali metal ions. *Acta physiol. scand.* 1975, **94**, 77-84.
- KUPPERMAN, A. S., B. ALTURA and J. CHEZAR, Action of procaine on calcium efflux from frog smooth muscle. *Nature (Lond.)* 1968, **217**, 673-675.

3322. O. M. N. I. ROXBOROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin reagent. *J. Biol. Chem.* 1951. 193, 265-275.
3323. W. H. C. and H. OETIKER, The action of caffeine on the activation of the contractile mechanism of muscle fibers. *J. Physiol. (Lond.)* 1968. 194 III 74.
3324. R. M. VENTURA and M. SZELANSKI, Vincristine-induced precipitation of microtubule proteins. *Proc. Natl. Acad. Sci.* 1969. 66, 498-499.
3325. J. M. and V. T. NACHREIN, Cell surface and pinocytosis. *J. Histochem. Cytochem.* 1965. 13, 64.
3326. J. and F. VYKOČIL, Possible role of Ca ions on the resting metabolism of frog sartorius muscle during potassium depolarization. *J. cell comp. Physiol.* 1966. 67 159-168.
3327. J. V. L. F. VYKOČIL, L. VYKOČIL and R. BERÁNEK, Potassium and caffeine induced increase of oxygen consumption in frog muscle and its inhibition by drugs. *Physiol. Bohemoslovaca* 1966. 11 277-284.
3328. R. P. M. B. FERNETI, E. D. JAMES and M. PETERS, Inhibition of catecholamine secretion and ion exchange in perfused cat adrenal glands by tetracaine and magnesium. *J. Pharmacol. exp. Ther.* 1971. 213, 463-471.
3329. LUTERBACH, G. Die Komplexeintrache Tätigkeiten. 4. Auflage, Stuttgart 1960.
3330. K. P. Erythrocyte membrane stabilization by local anesthetics and tranquilizers. *Biochem. Pharmacol.* 5, 15, 1753-1766.
3331. K. H. M. CHAN-WONG and S. MOTTEN, Membrane expansion by vincristine and strychnine. *Nature Med.* 1971. 211 22.
3332. K. P. and J. WINTER, Erythrocyte membrane stabilization by tranquilizers and anticholinergics. *Chem. Pharmacol.* 1966. 15 1737-1752.
3333. KURTZ, G., C. P. BLANCH and P. KRUPP Effect of local anesthetics on radioisotope binding in membranes. *Eur. J. Pharmacol.* 1970. 10, 91-100.
3334. T. A. P. and B. TAYMAN, Local Anesthetics. I. *Drugs Pharmacology in Medicine* 3rd Edition, ed. D. F. Palmer, New York, McGraw-Hill, 1963. 148-156.
3335. M. L., J. BRYAN, A. RILEY and D. MAXIA, Precipitation of proteins by vincristine and calcium ions. *Proc. Natl. Acad. Sci. (Wash.)* 1970. 66, 807-814.

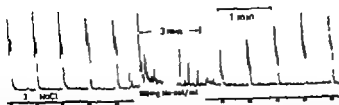
vinblastine competed with  $\text{Ca}^{++}$  for binding sites on these proteins providing support for the hypothesis that interference with  $\text{Ca}^{++}$  binding is also the basis for the inhibition of pinocytosis by this drug. On the present evidence concerning  $\text{Ca}^{++}$  and pinocytosis experiments indicate that organic bases act through an inhibitory effect on a  $\text{Ca}^{++}$  channel which couples the absorption of the chemical inducer in the membrane to the opening of channels.

This work was supported by the Swedish Cancer Society project number 167-K69-0-UX and the Faculty of the University of Lund, Sweden.

The following firms graciously donated drug samples. AB Leo, Helsingborg, Sweden (chlorpromazine-HCl and prochlorperazine methanesulphonate), AB Astra, Södertälje, Sweden (lidocaine-HCl 572), AB Bofors, Mölndal, Sweden (meprobamate-HCl, bupivacaine (Marcain®)), E. Lilly, Indianapolis, USA (vinblastine sulphate), Sandoz, Basle, Switzerland (thioridazine-HCl), AB Reck, Sweden (promethazine-HCl) and N-hydroxyethylpromethazine HCl (Aprobrin®), Ciba, Basle, Switzerland (dibucaine).

## References

- ACRIVER, J. and X. MACHIE, The action of calcium and of local anaesthetics on nerve cells, and its action during excitation. *J. Pharmacol. exp. Ther.* 1963, 140, 138-144.
- BALZER, H. and M. MAKINSON, The inhibition of the sarcoplasmic calcium pump produced by which react with its lipid component reserpine, prenylamine, chlorpromazine, meprobamate. *Schmidtschebergs Arch. exp. Path. Pharmacol.* 1968, 159, 151.
- BLAUSTEIN, M. P. and D. E. GOLDMAN, Competitive action of calcium and procaine on lobes. *J. gen. Physiol.* 1966 a, 49, 1043-1063.
- BLAUSTEIN, M. P. and D. E. GOLDMAN, Action of anionic and cationic nerve-blocking agents: Evidence and interpretation. *Science* 1966 b, 153, 429-432.
- BRANDT, P. W. and A. R. FREEMAN, Plasma membrane: Substructural changes correlated with resistance and pinocytosis. *Science* 1967, 155, 582-585.
- BRANDT, P. W. and K. B. HENDIL, Perturbances of the plasma membrane of amoeba. In: *Biochem. Advances in Exp. Medicine and Biology* 1970, 7, 323-335.
- BRANDT, P. W. and K. B. HENDIL, Plasma membrane permeability and pinocytosis in *Chaos*. *C. R. Lab. Carlsberg* 1972, 38, 423-443.
- COOPER, B. A. Qualitative studies of pinocytosis induced in *Amoeba proteus* by simple cations. *Lab. Carlsberg* 1968, 36, 383-403.
- FEDOTCH, M. B. Reaction of local anaesthetics with phospholipids. A possible chemical basis for action. *J. gen. Physiol.* 1964, 48, 357-374.
- HANSSON, S. E., G. JOHANSSON and J.-O. JOSEFSSON, Oxygen uptake during induced pinocytosis in *proteus*. *Acta physiol. scand.* 1968, 73, 491-500.
- HAUSER, H. and R. M. C. DAWSON, The displacement of calcium ions from phospholipid models by pharmacologically active and other organic bases. *Biochem. J.* 1968, 109, 909-916.
- HENDIL, K. B., Ion exchange properties of the glycocalyx of the amoeba *Chaos*. *Chaos* and its role in pinocytosis. *C. R. Lab. Carlsberg* 1971, 38, 187-211.
- JAANUS, S. D. E. MILLER and R. W. RUMR, The analysis of the inhibitory effect of local anaesthetic propranolol on adrenomedullary secretion mediated by calcium or acetylcholine. *Brit. J. Pharmacol.* 1967, 31, 319-330.
- JOHNSON, C. L. and A. SCHWARTZ, Some effects of local anaesthetics on isolated mitochondria. *J. Pharmacol. exp. Ther.* 1969, 67, 363-373.
- JOSEFSSON, J.-O., Induction and inhibition of pinocytosis in *Amoeba proteus*. *Acta physiol. scand.* 1973, 73, 481-490.
- JOSEFSSON, J.-O., N.-G. HOLMER and S. E. HANSSON, Membrane potential and conductance during pinocytosis induced in *Amoeba proteus* with alkali metal ions. *Acta physiol. scand.* 1975, 94, 273-283.
- KUPFERMAN, A. S., B. ALTURA and J. CHEZAR, Action of procaine on calcium efflux from frog skeletal muscle. *Nature (Lond.)* 1968, 217, 673-675.



The record shows the response to 0.3 M NaCl before and after the application of sodium salicylate, 100 mg/ml, on the tongue for 3 min. The record demonstrates that the taste response is depressed after salicylation.

### Methods

Male Sprague-Dawley rats were used. They were anesthetized with Hypnorm (Leo), which is a mixture of narcotic analgesic, 0.2 mg/ml of fentanyl, and a tranquilizer 10 mg/ml of flazepam, orally adapted for veterinary use. The initial dose of 0.5 ml/kg b. w. was injected i.m. The animals were intubated with heparinized, Medvetal containing 60 mg sodium heparin/ml. The right chorda palmaris nerve, which is a branch from the facial nerve to a trigeminal branch, the lingual proper nerve, was dissected. It mediates taste sensations from the anterior part of the tongue. The activity of the nerve is recorded under mineral oil, amplified and displayed on an oscilloscope. It was then recorded on a recorder, Stortz I, Varian Assoc., as a continuous trace. The tongue was stimulated with solutions of 0.3 M NaCl, 0.3 or 0.5 M sucrose, 0.02 M acetic acid and 0.02 quinine hydrochloride for about 3 s, cooled and followed by tap water at the same temperature. The device used to apply the taste stimuli already been described in detail (Ardreth *et al.* 1971). The taste solutions are heated in temperature-controlled water bath. The flow of these solutions was regulated through magnetic valves operated by timer circuits. The timing and sequence of the stimuli could be programmed. This technique allows reproducible, well-controlled stimulation of the taste buds.

Aspirin (acetyl salicylic acid) and sodium salicylate have been tested. When aspirin is used, 72 mg/ml and 52 mg/ml (hydroxymethyl) benzoates were made up in 1 ml distilled water. The solution is adjusted to pH 7.4. The sodium salicylate has used locally was dissolved in water 50, 75, 100, 200, 500 mg/ml. For intravascular infusion it was dissolved in Hemacel (a plasma substitute) or in Ringer's solution. Three different concentrations were used, 10, 25 and 100 mg/ml with wet. Lidocaine was also used. Before we had access to a water-soluble substance, through the courtesy of Dumas, it was dissolved in dimethyl sulfoxide or alcohol or in a mixture of these two. When water-soluble lidocaine was available, it was dissolved in concentrations of up to 30 mg/ml water or Ringer's solution.

The systemic blood pressure was recorded in one femoral artery. For direct infusion into the tongue of drugs, the right external maxillary artery was cannulated. The tip of the catheter was placed close to peripheral to the branching of the lingual artery. This left the blood supply to the tongue undisturbed. It allowed direct infusion of substances into the vascular bed of the tongue. The infusion was made with a syringe pump (Holter RD 074) the speed of which was monitored on the simultaneously recorded taste response. With three-way valves in the loop, different kinds of solutions could be selected. To prevent spasm at the catheter we added 100 I.U. of heparin/ml solution.

### Results

The effect of salicylates on the taste response has been studied with 3 different methods of application. Fig. 1 shows an example of the effect of topical application to the tongue. The responses to repeated stimulation with 0.3 M NaCl were recorded before and after the application of 100 mg sodium salicylate/ml for 3 min. Fig. 1 shows that after the application the response to the salt solution was depressed. It can be noticed that the salicylate solution itself increased the neural activity of which only the initial and last parts have been included in Fig. 1. These experiments were also repeated with solution of aspirin which was

## Depression of Taste Responses by Local or Intravascular Administration of Salicylates in the Rat

By

G HELLEKANT and V GOPAL<sup>1</sup>

Received 18 April, 1975

---

### Abstract

HELLEKANT G and V GOPAL. *Depression of taste responses by local or intravascular administration of salicylates in the rat* Acta physiol. scand. 1975 95 286-292.

The effects of aspirin and sodium salicylate on the taste response in the chorda tympani nerve have been studied during stimulation of the taste receptors in rats with salty, sweet and bitter-tasting solutions. Three methods of administering the drugs were used: locally on the tongue, intravenously into the femoral vein and intraarterially close to the branching of the lingual artery. It is observed that salicylates, given in any of these three ways, depressed the response to taste stimuli. This is discussed in relation to present views on the action of salicylates.

---

In an earlier series of experiments in which we studied the relationship between taste sensitivity and blood flow through the tongue in the rat we had problems with thrombocyte aggregation (Hellekant 1971). We were advised to give salicylate to prevent this. However, when we then tried to record a response to taste stimulation of the tongue we failed to record any response. We soon suspected that this failure was related to the salicylate we injected. Salicylates are capable of alleviating certain types of pain (Goodman and Gilman 1970). Some of the pain relief seems to be the result of an effect on peripheral receptors (Collier 1969). Thus it had been reported that salicylates in dog block apparently pain stimulation of certain visceral chemoreceptors evoked by intraarterially injected bradykinin (Lim *et al.* 1964). Further Scott (1968) concluded that topical application of aspirin to the dentinal receptors in cats promptly inhibits both the steady-state discharge and the response to brief heat stimulation. However these earlier reports describe the effect of salicylates on receptors responding to noxious stimulation. No report has suggested an effect of salicylate on receptors responding to non-painful stimuli such as weak salt solutions on the tongue. Our unwanted finding was therefore surprising. It seemed worth while to explore this unexpected effect of salicylates in more detail. A short preliminary report has already been made (Hellekant 1971).

<sup>1</sup> On leave from the Institute of Neurology Madras Medical College and General Hospital, Madras, India.

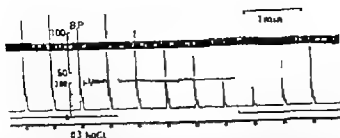
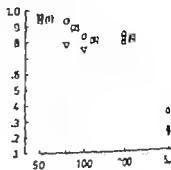


Fig. 4. The effect of infusing sodium salicylate into the tongue on the taste response to 0.3 M NaCl as recorded. The top trace illustrates the systemic blood pressure, the middle one the taste response and the bottom one indicates the rate of infusion. The record shows that after about 1.5 mg there is definite diminution of the taste response, while the effect on the blood pressure is small.

However, as shown by Fig. 3, a fairly high dose of salicylate had to be given when the substance was infused systemically. General effects of salicylate like transcapillary bleeding, changes in systemic blood pressure, etc. were frequently observed. In order to avoid these effects and to study the effect of intravascularly administered salicylates further we infused the substances via the external carotid artery directly into the tongue, as described in the methods. In this case a much lower dose gave an effect. Fig. 4 shows an example of this. A solution containing 10 mg sodium salicylate/ml solution was infused at a rate of 300  $\mu$ l/min. After about 1.5 mg there was a definite diminution of the taste response. Provided that the dose was not too large, the taste sensitivity usually then returned to the previous level when the infusion stopped. The cumulative effect observed during general systemic administration was less evident with this technique, but could be observed. The top trace in Fig. 4 shows the systemic blood pressure recorded in one femoral artery. This dose of salicylate did not affect it.

An effect of salicylates on peripheral receptors has been demonstrated by other authors (Van 1966, Scott 1968). Since it is now generally thought (*cf.* Vane 1973) that salicylates exert their effects via an inhibition of prostaglandins, we decided to study the effect of a substance which has a similar analgesic effect as salicylates but which is a stronger inhibitor of prostaglandins. Indomethacin was chosen because it has been described as being at least 100 times more potent than aspirin in its inhibition of prostaglandins. We have studied the effect of indomethacin on the taste response after topical as well as intravascular application. In 13 rats we tried infusion of solutions containing 2.5, 5, 10 and 50 mg indomethacin/ml at infusion rates varying from 100 to 300  $\mu$ l/min. We found that it was necessary to infuse about 2 mg at an infusion rate of not less than 1 mg/min to cause a depression of the taste response. An example of recording obtained during infusion with 5 mg indomethacin/ml solution at a rate of 300  $\mu$ l/min into the tongue is shown in Fig. 5. This record shows that no effect on the taste sensitivity was elicited although about 1.7 mg of indomethacin was infused. Further in 6 rats we applied indomethacin on the tongue in a concentration of 50 mg/ml. In 2 rats we used water-soluble indomethacin but observed no effects on the taste response. In 4 rats we used the usual commercially available substance which was dissolved in alcohol or dimethyl sulfoxide. In no case did we record an effect on the taste response.

Fig. 2. The ratios between the responses before and after sodium salicylate for 3 min on the tongue have been plotted against the concentration of salicylate used. The number of animals in each set of data is indicated by the figures in brackets. 3 different taste stimuli were used, 0.3 M NaCl ( $\square$ ), 0.02 M citric acid ( $\circ$ ) and 0.02 M quinine hydrochloride ( $\nabla$ ). The diagram indicates that a definite suppression of the taste sensitivity occurred at 100 mg salicylate/ml.



rinsed over the tongue. The taste response was depressed in a similar way as shown in Fig. 1. In 10 rats we studied the dose-effect relationship by applying sodium salicylate at different concentrations on the tongue for 3 min. The results are illustrated in Fig. 2, where the relationship between the responses before and after the salicylate is expressed as a ratio. The peak responses have been used. The first 4-5 responses of each stimulus after salicylate have been used. Fig. 2 shows that the responses to all stimuli used were depressed. Since we already knew that salicylate in higher concentration depresses the taste response, our interest focused on the concentration of salicylate that was necessary to cause a depression. One hundred mg/ml was considered to be a "critical" concentration. Thereafter we applied this concentration to all animals except the first one. From Fig. 2 it can be seen that the concentration that definitely causes a depression is somewhere between 100 and 200 mg. A significance test, statistics for two means, using the ratios between the responses before and after salicylate, gave a significant effect of 100 mg salicylate/ml solution ( $p < 0.05$ ) on the tongue. The depression caused by 500 mg/ml was strong and lasted for more than 30 min in spite of a continuous rinse of the tongue with water.

It seemed of interest to see if salicylates applied from within the taste buds would have an effect on the taste receptors. Fig. 3 shows an example of such an experiment. In this we infused the drug through one femoral vein. The animal received in total 200 mg aspirin/kg b.wt. In Fig. 3 the first bottom mark indicates the onset of infusion and the second bottom mark indicates the termination. In this rat after about 100 mg/kg b.wt. a significant decrease of the taste response could be recorded. A slight trace of hypersensitivity is also seen before the drug is administered.

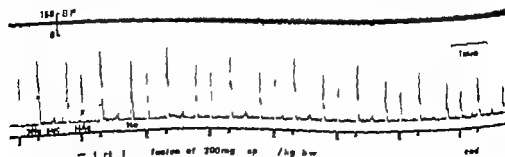


Fig. 3. The effect on the taste response of infusion of 200 mg aspirin/kg b.wt. is shown. It shows a decrease in taste sensitivity as the result of aspirin.



are then thought to work near the spot where they were released, giving rise to noxious reactions. Thus salicylates are supposed to depress only the responses from receptors activated by noxious stimulation and not by "normal" stimulation. This view makes our finding that salicylates diminish the sensitivity of the taste buds to a stimulation rather unexpected.

There appears to be two possible explanations. Either prostaglandins are in some way involved in the generation of the taste responses from the taste buds, or the effects of salicylates on these receptors differ from what at present is regarded as their mode of action on receptors (Collier 1971). For this reason we tested indomethacin, known to be a powerful inhibitor of the release or synthesis of prostaglandins. It is at least 10 times more potent *in aspirin*. Thus Vane (1973) reports data which suggest that it is 17 to 2140 times more potent as an inhibitor of prostaglandin synthesis. Our results show that indomethacin was able to suppress the taste sensitivity but the concentration needed was not lower than that of salicylates. It is possible that a difference in ability to penetrate the tissue may have played a role, but the fact that when it did have an effect, the latency was about the same for salicylate makes it more probable that prostaglandins have nothing to do with the generation of the taste response. This conclusion is also supported by a brief experiment with prostaglandin  $F_{2\alpha}$  in which 33  $\mu$ g and 63  $\mu$ g were infused into the tongue over 2 min in two separate experiments. A strong effect on the blood pressure was observed but no effect on the taste response. Prostaglandin  $E_2$  would have been a better choice, because it has been used as a pain-producing agent, but was not available. However from the data described we conclude that the action of salicylates on the taste receptors differ from what at present is regarded as its mode of action on receptors (Collier 1971, Vane 1973).

There are a number of effects of salicylates which can be regarded as possible explanations of our observations. Salicylates have general effects on metabolic processes (Goodman and Gilman 1970). They uncouple oxidative phosphorylation (Smith and Smith 1966), as a result of which, a number of adenosine triphosphate dependent reactions are inhibited. This gives rise to an increased demand for oxygen, as a result of the enhanced oxidation accompanying the relative inefficiency of the phosphorylating mechanism. Since the taste cells have a high demand for oxygen (Hellekant 1971) the decrease in sensitivity described might have been the result of an inability to satisfy this increased demand. The phase of hypersensitivity during the initial stage of salicylate administration supports this, because it is a general feature of a receptor that its threshold diminished initially when its ability to maintain its membrane potential deteriorates, as a result of hypoxia, for example. In summary this study shows that salicylates after topical application or intravascular administration to the tongue diminish and finally abolish the response of the taste receptors to taste stimulation. Experiments with indomethacin, known to be a more powerful prostaglandin inhibitor indicate that this effect of salicylate on the taste receptors is probably correlated to its effect on prostaglandins. This effect has been regarded as the mode of action of salicylate when suppressing the response from other peripheral receptors.

This study was supported by grants from Malttrycksstillsättningsrådet, Kung och Alice Wallenbergs Stiftelse, Almgrens Bergsunds Stiftelse and the Swedish Medical Research Council grant No 2447.

The supply of water-soluble indomethacin from Dumex Läkemedel AB is gratefully acknowledged.

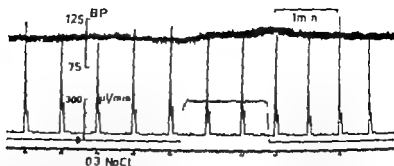


Fig. 5. Indomethazin, 5 mg/ml, was infused into the tongue at the same rate as in Fig. 4. The total given was about 1.7 mg. No depression of the taste responses was observed.

Since the concentrations of indomethazin we used without obtaining any effect were the same as those of salicylate, and since it is claimed that indomethazin is at least a 10% (Vane 1973) more efficient inhibitor of prostaglandin than aspirin, we conclude that the effects of salicylate described here on the taste sensitivity were not the result of its effect on prostaglandins.

### Discussion

This discussion will first focus on the dose of salicylates used and the possibility that the depression of the taste sensitivity observed might have been the result of a general intoxication.

Salicylates were formerly regarded as harmless drugs. This opinion has changed since salicylates are still widely used. In man the therapeutic maximum dose seems to be 100 mg/kg b.wt. and day (Goodman and Gilman 1970). In rat the  $LD_{50}$  is 500 mg/kg intraperitoneally (Ichniowski and Hueper 1946) and 125 mg/kg blocks bradykinin-induced vasodilation (Blane 1967). From these figures it seems to us that with the doses we used for the administration a general effect can be suspected as underlying the depression of taste sensitivity described here. However this would not explain the depression observed after local application to the tongue, when only a few mg could have entered the body.

The strong effect of local application is somewhat surprising, because the taste buds are known to withstand all kinds of toxic substances. The number of substances which penetrate the epithelium of the tongue is limited (Mistretta 1971). The present results indicate a rapid absorption of salicylates through the epithelium of the tongue similar to the rapid absorption through the intact skin (Goodman and Gilman 1970). Judged by the almost immediate discharge from the taste cells on the topical application of salicylates and the slower effect of infusion, it appears that the taste cells are the main target for salicylate and not the afferent fibres. This is supported by the findings of Lim *et al.* (1964) and Scott (1968) that the nerve impulse conduction in peripheral nerve is not affected by aspirin.

Previous studies (Lim 1966, Scott 1968) have demonstrated an effect of salicylates on the response to noxious stimulation. The present opinion seems to be (cf. Collier 1971, Vane 1973) that salicylates exert this effect via a blocking of the synthesis or release of prostaglandins, which are known to increase locally as the result of *in vivo* inflammation.

## Kinetics of the Glomerular Ultrafiltration in the Rat Kidney An Experimental Study

By

Ö. JÄLLSKOG, L. O. LINDBOM, H. R. ULFENDAHL and M. WOLGAST

Received 18 April 1975

### Abstract

JÄLLSKOG, Ö., L. O. LINDBOM, H. R. ULFENDAHL and M. WOLGAST *Kinetics of the glomerular ultrafiltration in the rat kidney. An experimental study* Acta physiol. scand. 1975. 95. 293-300.

quantitative relation between the driving forces over the glomerular membrane and the glomerular mass flow, on the one hand, and the single glomerular filtration rate (SNGFR), on the other is still absent. Micropuncture measurements on Sprague-Dawley rats made it possible to calculate the net driving force over the glomerular membrane. The single glomerular plasma flow was determined from GFR and the single nephron filtration fraction (SNFF). The effective plasma flow was measured with H<sub>2</sub> for total kidney and for superficial nephrons. The mean glomerular capillary pressure was found to be 61.6 mm Hg. The results indicate a net driving force of about 13 mm Hg at the distal end of the glomerular capillary. SNGFR was found to be 14.1  $\mu$ l/min/100 g. SNFF amounted to about 0.27. The filtration fractions determined with the PAH method were in the same range. The results indicate filtration equilibrium, in contrast to those of Brenner *et al.* from measurements on normal Wistar rat strains. The same fractions seemed to be the same in all glomerular populations. It is clear that the SNGFR is mass dependent. Our earlier findings of homeoregulation of the blood flow through the outer glomerulus was also confirmed.

While the seeping properties of the glomerular membrane have been extensively studied, information about the hydrostatic and oncotic forces operative over the glomerular membrane and about the glomerular plasma flow and the filtration fraction are still scanty. With the introduction of the servo-milling micropipette technique by Wiederhielm *et al.* (1964) it became possible to record the hydrostatic pressures within the superficial renal microcirculation. The glomeruli are usually not located in the superficial layer of the kidney in most Wistar strains with superficial glomeruli. Brenner *et al.* (1971) were able to perform direct punctures of the glomerular capillaries. They found a glomerular capillary pressure of about 45 mm Hg, a pressure which has since been confirmed by several workers (Brenner *et al.* 1972, Robertson *et al.* 1972, Blantz *et al.* 1972). This pressure is considerably lower than that obtained by the indirect stop-flow technique on the Sprague-Dawley rat (Gertz *et al.* 1966, 1969, Koch *et al.* 1967, Krause *et al.* 1967, Andrucci *et al.* 1971). Brenner

## References

- ANDERSSON, I. G. HELLEKANT, R. LARSSON, C. MOLANDER and L. STRÖM, A system for normally automatically controlled gustatory stimulation. *Med. Biol. Engin.* 1971 **9**, 715-717.
- BLANTZ, E. Blockade of bradykinin induced nociception in the rat as a test for analgesic drugs with preference to morphine antagonists. *J. Pharm. Pharmacol.* 1967 **19**, 367-373.
- COLLIER, H. O. J. Prostaglandins and aspirin. *Nature (Lond.)* 1971 **232**, 17-19.
- GOODMAN, L. S. and A. GILMAN, *The pharmacological basis of therapeutics*. The Macmillan Co. 1970 IV ed. 1794 p.
- HELLEKANT, E. The effect of changes in the blood flow to the tongue on the chorda tympani response in the rat. *Acta physiol. scand.* 1971 **82**, 145-153.
- HELLEKANT, G., The effect of acetylsalicylic acid on the taste response of the rat. *Proc. int. Union Physiol.* 1971 **9**, 715.
- ICENBOWER, C. T. and W. C. HUXTER, Pharmacological and toxicological studies on salicylates. *J. Pharm. Ass.* 1946 **35**, 223-230.
- LEE, R. K. S., *Salicylate analgesia*. In the salicylates, a critical bibliographic review 1966. John Wiley & Sons, Inc. New York.
- LEE, R. K. S., F. GUZMAN, D. W. RODGERS, K. GOTO, C. BRAUN, E. D. DECKERSON and R. J. E. Site of action of narcotic and non-narcotic analgesics determined by blocking bradykinin-induced pain. *Arch. int. Pharmacodyn.* 1964 **152**, 25-38.
- MISTRETTA, C. M. Permeability of tongue epithelium and its relation to taste. *Amer. J. Physiol.* 1962 **220**, 1162-1167.
- SCOTT, D. Jr. Aspirin action on receptor in the tooth. *Science* 1968, **161**, 180-181.
- SMITH, M. J. and P. K. SMITH, *The salicylates*, a critical bibliographic review. John Wiley & Sons 1966. New York.
- VANE, J. R., Inhibition of prostaglandin biosynthesis as the mechanism of action of aspirin-like drugs. *Bract.* 1973 **9**, 395-411.

In the second series the total and single nephron GFR as well as the total and single nephron PAH clearance were measured in addition to pressure measurements in the superficial, arcuate and tubular levels. The single nephron PAH clearance was measured by quantitative sampling in an oil-blocked tubular segment. These segments are identified by injections of fluorescent green through the proximal renal papilla into proximal tubular segments. This papilla remained in position under the sampling oil to control an adequate section. The volume of the sample was measured from the length in contact with capillaries (Microcups, 0.5 µl, Drexelbrook).

All experiments were performed during normo- and hypotensive conditions. The latter as induced by a clamp between the right and left renal arteries.

The radioactivity of the 125-I PAH and the 3-H Inulin used was measured in a liquid scintillation counter (LKB LS-2300).

In the third series the filtration fraction of single nephrons as determined, 125-I labelled albumin as well as the fraction as calculated from the concentration of radioactivity in plasma samples from the sampling points as related to the concentration of the activity in systemic plasma. The sampling of blood from sampling points proved to be very difficult in the adult, full-grown Sprague-Dawley rats. To prevent lung oil emboli all animals were heparinized with an intravenous injection of 2 000 I.U. heparin/kg b.wt. All samplings were performed within one hour after the injection of the heparin. The tip diameter of the cannula used was about 12 µm and the cannulae were filled with coloured mineral oil except for the very tip, which was filled with coloured heparin-saline solution. All punctures were made at a relatively small angle, directly into a bifurcation point or into a first order branch with the tip pushed forward into the wall of the vessel. The coloured heparin-saline solution was then injected by syringe to make sure that the puncture was purely intravascular. All punctures at which the dye leaked out into the tubules or the interstitium were discarded. The blood was collected by spontaneous flow or by sampling against slight counter pressure.

By clamping the counter pressure with the syringe the operator regulated the speed of the streaming of the oil into the tip of the cannula to be fairly constant. In some control experiments the pressure was measured just proximal to the sampling papilla and showed no divergence from that in intact vessels points, arcuate vessels or the first order branches not merely showed signs of vasoconstriction with subsequent reduction in the sampling rate, in such case the samples were discarded. After completion of the sampling cannulae were sealed with the oil superheating the lutealys. The samples were then put into microcups (Microcups, 0.5 µl Drexelbrook), sealed at one end by burning, and centrifuged for 2 min at 8 000 g. The volume of the plasma and red cells was determined, thereafter the samples are analysed in a gamma-counter. The hematocrit of the walling point blood as well as of the systemic blood was also calculated. Some variables were not used in the determination of the filtration fraction, though essentially the same values would have been obtained.

In the fourth series of experiments the effective driving force over the glomerular membrane was changed by applying counter pressure on the tubular side of the membrane. This was achieved by continuous injection of oil into a proximal segment until the desired intratubular pressure was obtained. The pressure was measured with pressure recording cannulae inserted in an early proximal tubular segment. Tubular fluid was sampled in a conventional way via a pipette inserted just proximal to the oil block, at such a depth as to keep the oil column fixed. While another operator controlled the tubular pressure by adjusting the speed of the continuous oil injection. The sampling times were comparatively long (4-6 min) to eliminate an effect of transient changes in filtration when inducing the counter pressure.

## Results

In the first series of experiments the results from the pressure measurements are depicted in Table I. At a mean arterial pressure of 118 mm Hg the glomerular capillary pressure averaged  $62.6 \pm 3.2$  mm Hg (mean  $\pm$  S.D.). The pressures in the early proximal tubules blocked with oil were  $37.7 \pm 3.9$  mm Hg, which by adding the systemic oncotic pressure of 20.3 mm Hg (6% protein) would mean a glomerular pressure of 58.0 mm Hg. A more precise calculation also taking into account the actual albumin-globulin ratio would mean an oncotic pressure of 22 mm Hg (Kilickog and Wolgast 1973). The results obtained by the stop-flow method thus corroborate

and coworkers (1969-1971 b) were also able to withdraw blood samples from the distal end of the efferent arterioles, which are seen on the surface of the kidney branching into the peritubular network with a stellate appearance (welling points or star vessels). By comparing the protein concentration in these samples with that in systemic plasma the filtration fraction could be calculated and thereby also the oncotic pressure in the distal end of the glomerular capillaries. Determinations of the single nephron filtration rate (SNGFR) and the renal glomerular plasma flow also allowed for an evaluation of the dynamics of the glomerular ultrafiltration. The results suggested that the mean net driving force is comparatively low and highly dependent on the plasma flow due to the fact that a filtration equilibrium is achieved at the distal end of the glomerular capillaries.

The occurrence of superficially located glomeruli even in the Sprague-Dawley rat, though very rare, allowed the same measurements to be made in the present study as those of Brenner and coworkers. Samples of welling point blood also permitted determination of the filtration fraction. The results show a much higher glomerular capillary pressure, about 63 mm Hg, and a single nephron plasma flow of more than twice that obtained in mutant Wistar rat.

### Methods

All experiments were performed on male Sprague-Dawley rats weighing between 250 and 350 g. The rats were fed on a standard diet (Antikrex, Sweden) and had food and water *ad libitum*. They were anesthetized with Inactin® (Chemical Fabr. Promonta, Hamburg, West Germany) intraperitoneally at a dose of 120 mg/100 g b.wt. and placed on a servo-controlled heating pad. The rats were tracheotomized and the left femoral artery and femoral vein were catheterized. The left kidney was exposed via a subcostal incision and placed in a Lucite cup, whereafter the ureter was cannulated. In most cases the dorsal side of the kidney was used for micropuncture, but in some few cases the ventral side was used. During the micropuncture the kidney was superfused with warmed mineral oil.

The experiments were divided into four series. In the first series of 9 rats the hydrostatic pressure in the tubules and the cortical vascular tree were measured, including direct measurements on glomerular capillaries. Such punctures are only possible when a glomerulus is located on the surface of the kidney, which is rare in the Sprague-Dawley rat. This series of experiments therefore comprised animals in which such superficial glomeruli were found incidentally. Superficial glomeruli, when present, are most often located on the ventral side of the kidney. For the purpose of pressure measurement, a servo-controlled pressure measuring device as designed by Wiederhielm (1964) and modified by I. Taghetti *et al.* (1971) was used. The punctures were performed with sharpened glass capillaries with an outer diameter of 100 µm. The glomerular capillary pressure was measured by means of direct punctures of the superficial glomerular capillaries and was verified by injection of Lissamine green into the glomerular capillary tuft. All micropunctures were performed with a glass needle which a leak gap into the interstitium, the tubules or Bowman's capsule was established. The pressures in Bowman's space were recorded by punctures deep into the glomerulus and verified by injections of dye, which under appropriate conditions filled Bowman's space and emptied out into the proximal tubule. The hydrostatic pressure in the distal end of the efferent arteriole was obtained by punctures at the welling points, which were easily identified on the surface of the kidney.

The glomerular capillary pressure was also measured indirectly by the stop-flow method as described by Gertz (1966). For this purpose an early proximal tubule was identified and punctured with a glass cannula (about 10 µm) filled with coloured mineral oil. The intratubular pressure was continuously monitored by means of a second cannula inserted proximal to the first one. The oil block was supplied until filtration had ceased and the pressure was stabilized. The glomerular capillary pressure was calculated by adding this "stop-flow" pressure to the colloid osmotic pressure of the systemic plasma. A protein concentration of 6% would then correspond to a pressure of 20-21 mm Hg as calculated by the formula of Landis and Pappenheimer (1963). This series was completed with some control experiments concerning the validity of stop-flow measurements. The oil blocks were applied to distal, late proximal, mid-proximal and early proximal tubules and the stop-flow pressure was recorded just above the oil column.

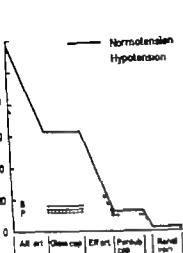


Fig. 1

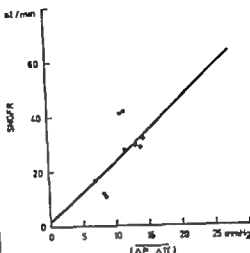


Fig. 2

Hypotensive pressures in the superficial renal vasculature, Bowman's space and the proximal tubules. Upper line refers to normotensive conditions and the lower to hypotension of about 80 mm Hg. In the curve the glomerular capillary pressures were investigated indirectly by the "step-flow" method only.

2. Relation between the change of the mean driving pressure,  $\Delta P - \Delta \pi$ , and the SNGFR. For each pair of intercept on the ordinate and the slope were calculated. The line drawn represents the mean of the individual lines.

0.28. The superficial SNGFR was  $14.1 \pm 1.9$  nl/min/100 g, and the single nephron effective plasma flow (SNEPF)  $56.4$  nl/min/100 g, which will mean a filtration fraction of 0.26. When the perfusion pressure in the renal artery was reduced from 124 mm Hg to 77 mm Hg, the total kidney PAH clearance was only reduced to 90% of the control value and the reduction took place in total GFR, i.e. these variables will be autoregulated. In contrast, the superficial SNGFR showed a more pronounced reduction, down to 10.4 nl/min/100 g, i.e. 73.8%. Essentially the same reduction was noted for SNEPF. The SNEPF was reduced to 79.6%.

The filtration fraction estimated from the relation between protein concentration in samples withdrawn from welling points and the concentration in systemic plasma, as undertaken in 9 rats in the third series, was  $0.27 \pm 0.06$  (mean  $\pm$  S.D.). Essentially no change occurred ( $0.28 \pm 0.04$ ) when the perfusion pressure was reduced from a mean value of 117 mm Hg to 81 mm Hg. Further reductions in perfusion pressure were followed by decreases in the filtration fraction.

In the fourth series II was found that the SNGFR was linearly related to change of the mean driving pressure,  $\Delta P - \Delta \pi$ . This result was obtained by determination of the SNGFR at two pressure levels. The first sampling was undertaken at about a normal pressure and the second one during elevated pressure. For the method of calculation of the mean driving pressure the reader is referred to Källskog *et al.* (1975 b); the glomerular plasma flow was assumed to be the same under both conditions. Fig. 2 shows the results from 18 pairs of

TABLE I. Hydrostatic pressures within the superficial tubular and vascular network in the rat. *n* refers to number of animals. Mean  $\pm$  S.D.

Arterial pressure mm Hg	Glomerular capillaries mm Hg	Proximal stop flow pressure mm Hg	Welling point pressure mm Hg	Peritub. cap. pressure mm Hg	Prox. tub. pressure mm Hg	Bowman's cap. pressure mm Hg
118 $\pm$ 7.3 <i>n</i> = 9	62.6 $\pm$ 3.2 <i>n</i> = 9	37.7 $\pm$ 3.9 <i>n</i> = 9	13.9 $\pm$ 1.2 <i>n</i> = 8	10.8 $\pm$ 1.2 <i>n</i> = 8	13.5 $\pm$ 1.3 <i>n</i> = 9	16.0 $\pm$ 2.6 <i>n</i> = 5

those arrived at directly. The stop-flow pressure in the second series (see Table II) amount to 40 mm Hg, thus essentially the same as in the first series. A systematic error might have been inherent in the stop-flow measurements. A small degree of filtration could still occur especially when the blockade was made in late proximal tubules. The pressures obtained in distal tubules and late proximal ones are considerably lower than those in the earlier experiment. The pressure in welling points in the two series amounted to 14 mm Hg. Within peritubular capillaries the pressure was 11–12 mm Hg.

In the tubular system the pressures within Bowman's space are slightly higher than in the proximal tubule, indicating a slight pressure drop which might be located to the neck of Bowman's capsule. The proximal tubular pressure was estimated at 13.5 mm Hg, with very small scattering. The data on hydrostatic pressures within the superficial vasculature and tubules are summarized in Fig. 1. The data under normotensive conditions were derived from the first series; the data from the second series would, however, be in complete concordance. For hypotensive conditions all data are derived from the second series, and it should be emphasized that the glomerular capillary pressure was only measured indirectly.

For the second series single nephron and total clearance of tritiated inulin and  $^{125}$ I-labelled PAH were obtained, in addition to the pressure data. The total GFR was 0.37  $\pm$  0.08 ml/min as calculated per 100 g b wt. The PAH clearance, which may be regarded as a measure of effective renal plasma flow, amounted to 1.33  $\pm$  0.19 ml/min 100 g, the filtration fraction

TABLE II. Hydrostatic pressures within the superficial tubules and the vascular network and data on single nephron glomerular filtration (SNGFR), single nephron PAH clearance (SNEPF), total blood flow (GFR) and total PAH clearance (EPF), TI/p inulin in the distal tubules and the single nephron determinations on each rat. Mean  $\pm$  S.D.

Arterial pressure mm Hg	Prox. tub. stop-flow mm Hg	Prox. tub. free flow mm Hg	Welling point mm Hg	Peritubular capillaries mm Hg	SNGFR ml/min 100 g	SNEPF ml/min 100 g
124 $\pm$ 4 77 $\pm$ 4	40.1 $\pm$ 2.7 30.4 $\pm$ 2.4	15.9 $\pm$ 0.9 12.1 $\pm$ 0.9	14.0 $\pm$ 1.7 11.2 $\pm$ 1.4	11.4 $\pm$ 1.8 —	14.1 $\pm$ 1.9 10.4 $\pm$ 1.4	56.4 $\pm$ 4.0 44.9 $\pm$ 13.1
	SNEPF		GFR ml/min 100 g	EPF ml/min 100 g	FF	TI, p inulin
124 $\pm$ 4 77 $\pm$ 4	0.26 $\pm$ 0.02 0.24 $\pm$ 0.06		0.37 $\pm$ 0.06 0.33 $\pm$ 0.11	1.33 $\pm$ 0.19 1.20 $\pm$ 0.29	0.28 $\pm$ 0.01 0.27 $\pm$ 0.03	3.6 $\pm$ 0.34 4.3 $\pm$ 1.27



The plasma flow measured with PAH is not representative for the outermost glomeruli. tubules belonging to these glomeruli pass far down into the renal parenchyma and will extract PAH from other regions than the outer cortex. It is thus concluded that the  $\text{PAH}$  method cannot be used for the measurement of plasma flow in superficial glomeruli. A very interesting finding is that the filtration fraction obtained by 1) the above mentioned nephron effective plasma flow method, 2) the total renal plasma flow and total GFR estimations, and 3) the welling point method, will show the same value of about 0.27. This strengthens the assumption of an equal filtration fraction in all the glomerular population. Since the filtration fraction is almost the same during both normo- and hypotension one is led to speak of an autoregulation of the filtration fractions. This is in good accordance with the results obtained by Brenner *et al.*

Brenner *et al.* concluded that the glomerular filtration rate is directly correlated to the glomerular plasma flow which is a consequence of a filtration equilibrium. As the present study appears to indicate a non-equilibrium, a pressure dependency rather than a plasma flow dependency might be expected. This problem has been evaluated further by Källskog *et al.* (1975 b). The effect of the driving pressure on the glomerular filtration rate is further supported by the results of the experiments in which a counter pressure was applied in the Bowman's space, and a linear relation between the mean driving force and the GFR was found. In hypotensive rats, however, the plasma perfusion rate was the dominant factor affecting GFR. Thus the kinetics of the glomerular filtration process is normotensive in mutant Sprague-Dawley rats are comparable with those of hypotensive Sprague-Dawley rats.

In earlier investigations (Källskog *et al.* 1975 a) it has been shown that the outer glomeruli do not autoregulate with respect to the blood perfusion rate. From SNGFR and the single nephron filtration fraction under normotensive and hypotensive conditions it was possible to calculate the blood flow of single glomeruli also in these series of experiments. The results indicate the same non-existence of an autoregulation of the blood flow in the superficial glomeruli.

### References

- LISSON, M. E. M., E. M. LIPMAN and C. W. GOTTSCHALK. Hydrostatic pressure in the rat kidney. *Amer. J. Physiol.* 1972, 223, 975-983.
- MORILLAS, V. J., H. ACOSTA, F. C. RAYSON, D. SELIGER. Effective glomerular filtration pressure and single nephron filtration rate during hydropexis, elevated arterial pressure, and acute volume expansion with isotonic saline. *J. clin. Invest.* 1971, 50, 2230-2234.
- LANZ, R. A. H., H. ACOSTA, F. C. RAYSON, D. W. SELIGER. Relation of distal tubular  $\text{NaCl}$  delivery and glomerular hydrostatic pressure. *Kidney Intern.* 1972, 2, 22-32.
- ROSE, R. K. N., K. FALCOW, R. KIMMITS and R. BERLINER. The relationship between peritubular capillary protein concentration and fluid reabsorption by the renal proximal tubule. *J. clin. Invest.* 1969, 44, 1518-1531.
- REINER, R. J. L. TAOY. Postglomerular vascular protein concentration. Evidence for a causal role in governing fluid reabsorption and glomerulotubular balance by the renal proximal tubule. *J. clin. Invest.* 1971, 50, 324-349.
- REINER, R. J. L. TAOY and T. M. DAUGHERTY. The dynamics of glomerular ultrafiltration in the rat. *J. clin. Invest.* 1971, 50, 1776-1780.
- REINER, R. J. L. TAOY and T. M. DAUGHERTY. Pressures in cortical structures of the rat kidney. *Amer. J. Physiol.* 1972, 223, 246-251.
- DEZEL, K. H., J. A. MANNION, G. BRAUN and H. E. FAGEL. Pressures in the glomerular capillaries of the rat kidney and its relation to arterial blood pressure. *Pflügers Arch. ges. Physiol.* 1966, 262, 343-374.

determinations. For each pair the intercept on the ordinate and the slope were calculated. The line drawn represents the mean of the individual lines.

The blockade means that the delivery to the distal tubules and thereby the macula densa region is zero. This would not seem to be of great significance, however, since the  $\text{SNGFR}$  as determined by proximal sampling corresponds to that obtained on sampling in the distal tubules as shown by Persson *et al.* (1974) in experiments on rats fed on the same kind of diet. The glomerular blood flow was assumed to be unchanged during the blockade.

## Discussion

Brenner and coworkers used rats of a mutant Wistar strain, because of the frequent occurrence of superficial glomeruli in these rats. However, the hydrostatic pressures within the glomerulus seem to differ from those found in the more commonly used non-mutant Wistar and Sprague-Dawley rats. In the Sprague-Dawley strain, which we have used, superficial glomeruli suited for micropuncture are found occasionally. It seems more reliable to use data from these kidneys, since the results can be compared with the data obtained in micropuncture experiments.

The glomerular hydrostatic pressures obtained in the present investigation are higher than those measured by Brenner *et al.* Most striking is the fact that the glomerular capillary pressure of the Sprague-Dawley rats exceeds that of the mutant Wistar rats more than 15 mm Hg. As the hydrostatic pressure in Bowman's space and the oncotic pressure do not differ between the two rat strains, the net driving force seems consequently to be higher in the Sprague-Dawley rats. (See also Allison *et al.* 1972.)

In this context the problem of the existence of an equilibrium or a disequilibrium regarding the filtration process must also be kept in mind. The mean net driving pressure under normotensive conditions can be calculated to be about 20 mm Hg, and during hypotension about 12 mm Hg. From the direct pressure measurements and the determination of the filtration fraction a driving pressure of about 13 mm Hg can also be calculated at the end of the glomerular capillary, which means that an equilibrium does not exist under normotensive conditions. During hypotension the end-capillary net driving pressure decreased to 0 mm Hg. The implication of the presented results differs from that of the Brenner group and will be discussed in detail in a forthcoming paper (Källskog *et al.* 1975 b).

The direct pressure measurements in Bowman's space and in the early proximal tubule showed a pressure difference of about 2.5 mm Hg and indicate a flow resistance at the junction between Bowman's space and the proximal tubule. This has to be taken into consideration when calculating the driving forces over the glomerular membrane. In this context it must also be pointed out that the directly measured intracapillary pressure is somewhat higher than that obtained by the stop-flow method. The more distally the stop flow point is measured, the greater the difference.

The single nephron effective plasma flow measurements with PAH during normotension and hypotension indicated an autoregulation of the renal plasma flow, which is not in accordance with the earlier findings of non-autoregulation in the outer parts of the renal cortex in the rat (Källskog *et al.* 1975 a). This difference is due to the fact that the single ne-

## Effects of Hypoxia of 10-45 Seconds Duration on Energy Metabolism in the Cerebral Cortex of Unanesthetized and Anesthetized Rats

By

KARIN NORBERG, BJÖRN QVISTORFF and BO K. SJÖGÅRD

Received 18 April 1975

### Abstract

NORBERG, K., B. QVISTORFF and B. K. SJÖGÅRD. *Effects of hypoxia of 10-45 seconds duration on energy metabolism in the cerebral cortex of unanesthetized and anesthetized rats.* Acta physiol. scand. 1975 95: 301-310.

Glycolytic and citric acid cycle intermediates, as well as organic phosphates, were measured in the cerebral cortex of unanesthetized rats following arterial hypoxia (administration of 4-8% O<sub>2</sub>) of 10 and 20 seconds. There were decreases in glucose-6-phosphate and fructose-6-phosphate, and increases in fructose-1,6-bisphosphate, dihydroxyacetone phosphate and 3-phosphoglycerate, even before pyruvate accumulated. An increase in the lactate concentration showed that there was an increased glycolytic rate, the data demonstrating that phosphofructokinase is activated. The glycolytic changes were accompanied and probably due to, minor changes in phosphocreatine, ATP, ADP and AMP. Experiments of anesthetized animals showed that hypoxia for 45 s was accompanied by signs of phosphofructokinase activation, even if tissue P<sub>CO<sub>2</sub></sub> was kept constant. It is concluded that, irrespective of the tissue CO<sub>2</sub> tension, hypoxia is accompanied by activation of phosphofructokinase which, at least initially is responsible for increased glycolytic rate.

Recent results (Duffy *et al.* 1972, Bacholard *et al.* 1974, Norberg and Sjögård 1975a and b) have provided new information on the cerebral metabolic changes that occur when animals are exposed to atmospheres containing low concentrations of oxygen ("hyoxic hypoxia"). The following tentative conclusions were drawn. Soon after the induction of hypoxia, there is an acceleration of glycolytic rate with accumulation of lactate and pyruvate, and increase in the lactate/pyruvate ratio. As a part of a generalized oxidation/reduction change there is also an increase in the malate/oxaloacetate ratio with a fall in oxaloacetate (OAA) concentration. The latter induces a shift in the aspartate aminotransferase equilibrium, resulting in an initial reduction in  $\alpha$ -ketoglutarate ( $\alpha$ -KG) level and a sustained reduction in aspartate. When hypoxia is prolonged, the elevated pyruvate concentration induces a shift in the aspartate aminotransferase reaction towards alanine formation, as well as an increased rate of

- GERTZ, K., M. BRANDIS, E. BRAUN-SCHUBERT and J. W. BOYLAN, The effect of saline infusion and hemorrhage on glomerular filtration pressure and single nephron filtration rate. *Pflügers Arch. ges. Physiol.* 1969 310 193-205.
- INTAOLLETTA, M., R. F. PAWULA and W. R. TOMPKINS, Pressure measurements in the mammalian microvasculature. *Mikroscop. Res.* 1970, 2, 212-220.
- KÄLLSKOG, Ö. and M. WOLGAST, Driving forces over the peritubular capillary membrane in the rat during antidiuresis and saline expansion. *Acta physiol. scand.* 1973, 89, 116-125.
- KÄLLSKOG, Ö., L. O. LINDBOM, H. R. ULFENDAHN and M. WOLGAST, The pressure-flow relations of different nephron populations. *Acta physiol. scand.* 1975 a, 94, 289-300.
- KÄLLSKOG, Ö., L. O. LINDBOM, H. R. ULFENDAHN and M. WOLGAST, Kinetics of the glomerular filtration in the rat kidney. A theoretical study. *Acta physiol. scand.* 1975 b, 95, 191-200.
- KOCH, A. M., T. DUMS, H. H. KRAUSE and B. OCHWADT, Intratubulärer Druck, glomerulärer Capillardruck und Glomerulumsfiltrat während Mannit Diuresis. *Pflügers Arch. ges. Physiol.* 1962, 265, 21-29.
- KRAUSE, H., T. DUMS, A. M. KOCH and B. OCHWADT, Intratubulärer Druck, glomerulärer Capillardruck und Glomerulumsfiltrat nach Furosemid und Hydrochlorothiazid. *Pflügers Arch. ges. Physiol.* 1962, 265, 80-89.
- LANDIS, E. M. and J. R. PAPPENHEIMER, Exchange of substances through the capillary wall. *Handbook physiology: Circulation II* Waverly Press, U.S.A. 1963 961-1034.
- PERSSON, E. and P. WRIGHT, Reduction of glomerular filtration rate by intrarenal feedback during  $\alpha$ -zalanide diuresis. *Fed. Proc.* 1974, 33, 347.
- ROBERTSON, C. R., W. M. DEEN, J. L. TROY and B. M. BRENNER, Dynamics of glomerular circulation in the rat. III. Hemodynamics and autoregulation. *Amer. J. Physiol.* 1972, 223, 1191-1200.
- WEIDENHILM, C. A., J. W. WOODBURY, S. KURTZ and R. F. ROSSIGNOL, Potassium pressures in the microcirculation of frog's mesentery. *Amer. J. Physiol.* 1964, 207, 173-176.

*Unanesthetized animals*

unanesthetized animals, weighing 200-220 g. preliminary operation was performed under 2% ether anesthesia, and tail artery catheter was inserted. After 1-2 hrs of recovery the rats were placed in the cylinder of the freeze-clamping apparatus (see Quasthoff 1975), and were kept there for 10-20 min. During that period of adaptation the cylinder was flushed with room air and measurements were made of blood pressure, rectal temperature and of arterial  $P_{O_2}$ ,  $P_{CO_2}$  and pH. The rats were then subjected for either 10 or 20 min by rapid switching to hypoxic gas mixture containing 10%  $O_2$ . In preliminary experiments, serial samples were drawn from the artery for determining the time of the changes in arterial gas tensions during the induction of hypoxia.

Controls were freeze-clamped at the end of the adaptation period, and the hypoxic animals at the end of the 10 and 20 periods of hypoxia. At the time of freeze-clamping, an arterial blood sample was drawn. The performance of the apparatus is such that the part of the rat positioned in the "trophy region" of the cylinder is cut free and freeze-clamped between precooled aluminum blocks in about 0.1 s. (For details of sampling procedure, see Quasthoff 1975.) The freeze-clamped slice, which contained the mid-part of the brain, was immersed in liquid nitrogen and later stored at  $-80^\circ\text{C}$ . Using buzz saw at  $30^\circ\text{C}$ , 10-12  $\mu\text{m}$  thick sections were then cut off from the surface of the brain sections that had been brought in contact with one of the freeze-clamping blocks. Cerebral cortical slices from these thin sections were used for tests.

*Anesthetized animals*

unanesthetized series of animals (hypoxic and control animals, the latter maintained at an arterial  $P_{O_2}$  of 180 mm Hg), the techniques used were similar to those described previously (Nörberg and Sjöberg 1974) and the reader is referred to that publication for details. The main features were as follows. Animals were intubated with 2-3 halothane and the animals were tracheotomized, intubated with carbon dioxide and connected to a respirator. At the time of tracheotomy the halothane anesthesia was removed and the animals were maintained on 70%  $N_2O$  and 30%  $O_2$  until hypoxia was induced. An arterial catheter was inserted to allow sampling of arterial blood and measurement of blood pressure. The rectal temperature, as measured in the rectum, was kept close to  $37^\circ\text{C}$ . The skin over the head was covered with polyethylene plastic (used for subsequent freezing of the tissue in vacuo). At the induction of hypoxia, the  $O_2$  concentration of the inspired air was decreased so as to give (final) arterial  $P_{O_2}$  of about 50 mm Hg, keeping the  $N_2O$  concentration constant. The gas mixture administered contained sufficient  $CO_2$  to increase arterial  $P_{CO_2}$  by 2-4 mm Hg during the hypoxia. After 45 min of hypoxia, the animals were killed by passing liquid nitrogen into the trachea. The frozen tissue was kept at  $-80^\circ\text{C}$ . Cortical tissue was then cut out, weighed and extracted at  $-22^\circ\text{C}$ .

*Anal techniques*

$P_{O_2}$  and  $P_{CO_2}$  in arterial blood were measured at  $37^\circ\text{C}$  with microelectrodes. Cortical tissue was dissected, sliced and extracted with HCl-carbonate at  $-22^\circ\text{C}$ . The following metabolites were analyzed (in some cases only selected number was studied): phosphocreatine (PCr), creatine, ATP, ADP, AMP, G-6-P, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-bisphosphate (FDP), dihydroxyacetone phosphate (DHAP), 3-phosphoglycerate (3-PG), pyruvate, lactate, citrate,  $\alpha$ -ketoglutarate ( $\alpha$ -KG), succinate, malate, glutamate, aspartate and NH $_4^+$ . The basic methods were those described by Lowry and Passonneau (1972) and analytical conditions have been given in previous communications (for references, see Folbergrove et al. 1974). The oxaloacetate concentrations were calculated from aspartate oxaloacetate transamination reaction, using an equilibrium constant of 6.7 (Krebs 1957). Metabolic differences were calculated using Aspin-Welch test or the nonparametric Mann-Whitney U-test, from the values when the group did not seem to follow normal distribution. The following levels are used: \*  $p < 0.05$ , \*\*  $p < 0.01$  and \*\*\*  $p < 0.001$ .

## Results

*Unanesthetized animals*

ten animals were provided with tail artery catheters, and cortical tissue was obtained for analysis by means of "freeze clamping" following exposure to about 8 and 6%  $O_2$ , respectively. Originally the gas with the lower concentration (6%) was administered to one group

CO fixation. As a result, there is an increased alanine concentration and an increase in pool size of tricarboxylic acid cycle (TCA) intermediates.

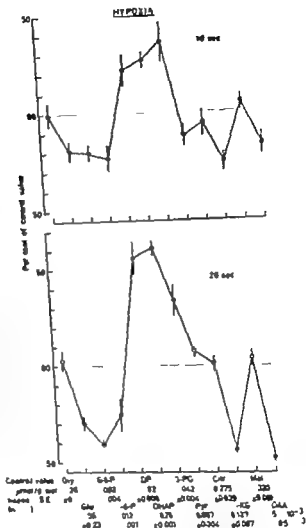
There have been some difficulties in defining the regulatory enzymatic reaction that is responsible for the accelerated glycolysis. Two previous studies were partly devoted to this problem (Duffy *et al* 1972, Bachelard *et al* 1974) but in none could evidence be obtained for an activation of phosphofructokinase (PFK) the enzyme which is considered to be the main regulatory control of glycolytic rate (Lowry and Passonneau 1964, 1966). The results of both groups of workers were consistent with the idea that, in hypoxia, pyruvate kinase may be regulatory. However by shortening the period of hypoxia to 45 s, or 1- and 45 s, and by allowing the  $P_{CO_2}$  to fall spontaneously we could show that an activation of PFK activity is involved in triggering an increased glycolytic rate (Norberg and Sjölö 1975 a). Our tentative explanation for the difference in results between that study and the previous one (Bachelard *et al* 1974) was as follows. Soon after the induction of hypoxia there is an imbalance between production and utilization of ATP. The consequences of this imbalance, *i.e.* a moderate increase in ADP (and  $P_i$ ), and a decrease in phosphoenolpyruvate, lead to PFK activation and increased glycolysis. However the activity of PFK is also decreased, relative to the activities of hexokinase and of pyruvate kinase, and this "inactivation" of PFK is accelerated by the development of tissue acidosis.

Before a completely satisfactory explanation can be given of the glycolytic events in hypoxia some further information is required. *Firstly* previous results were obtained in unanesthetized animals and since anaesthesia may blunt the tissue response to hypoxia, it is not desirable to study unanesthetized animals. *Secondly* no information is at hand about the metabolic changes occurring during the first 45 s period of hypoxia. Thus, tissue analyses performed at the very induction of tissue hypoxia might reveal larger changes in labile phosphates than are observed after 45 s in anesthetized animals. Such information is also essential for establishing that the increased pyruvate and lactate concentrations are consequences of increased glycolysis, and not just due to reduced pyruvate oxidation. Thus, if it could be shown that PFK is activated before pyruvate and lactate have accumulated, this problem would be solved. *Thirdly* when the arterial  $P_{CO_2}$  is allowed to fall during the hypoxia, there is in all probability an alkaline shift of intracellular pH during the first minutes (Norberg and Sjölö 1975 a). Since alkalosis is believed to stimulate glycolysis (Delcher and Stupp 1964) it becomes difficult to decide whether or not it contributes to PFK activation. Accordingly it would be desirable to establish that PFK activation occurs at constant CO<sub>2</sub> tension in the tissue.

The present experiments were designed to provide information on the above points. Using a "freeze clamping" technique (Qvistorff 1975) it was possible to obtain cortical tissue for analysis from unanesthetized animals, and to study hypoxia of only 20 sec duration. Activation of glycolysis at an unchanged tissue CO<sub>2</sub> tension was shown in anesthetized animals following a hypoxic period of 45 sec.

### Methods

The experiments were performed on male Wistar rats that had free access to water and rat pellets. There were two main series of animals—unanesthetized and anesthetized—and these are described separately.



2 Influence of hypoxia (administration of 6-8 O<sub>2</sub>) of 10-20 duration upon cerebral concentrations of glycolytic and cycle metabolites. Values are means  $\pm$  S.E. in per cent of the control values that are at the bottom. Filled symbols are values significantly different from the controls ( $p < 0.05$ ) retested used. Gly: glycine, Glc: glucose, G-6-P: glucose-6-phosphate, F-6-P: fructose-6-phosphate, FDP: fructose-1,6-bisphosphate, DHAP: dihydroxyacetone phosphate, 3-PG: 3-phosphoglycerate, Pyr: pyruvate, Lact: lactate,  $\alpha$ -KG:  $\alpha$ -ketoglutarate, Mal: malate, OAA: oxaloacetate.

a. The latter was measured in 4 control animals and in 4 animals exposed to 6% O<sub>2</sub> for 10 min. The blood glucose concentrations in these groups were  $10.2 \pm 0.3$  and  $9.5 \pm 0.4$   $\mu$ mol/g, respectively and the corresponding tissue to blood glucose concentration ratios were  $0.33 \pm 0.04$  and  $0.32 \pm 0.03$ , respectively. Thus, there were small, if any changes in the ratios. In the hypoxic animals, there were decreases in G-6-P, F-6-P,  $\alpha$ -KG and (calculated) OAA, and increases in FDP, DHAP and 3-PG. These results are entirely consistent with those obtained previously (Norberg and Sjöberg 1975 a and b) and demonstrate that, in unanesthetized animals, activation of PFK occurs already 10 s following induction of hypoxia (see Discussion).

Although there were signs of an activation of PFK at 10 s of hypoxia, there was neither increase in tissue lactate content at that time, nor significant elevations of lactate/pyruvate

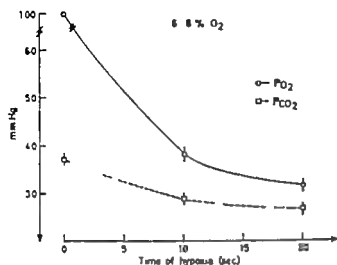


Fig. 1 Time course of changes in  $P_{aO_2}$  and  $P_{vO_2}$  during administration of 6-8%  $O_2$  to unanesthetized rats. Values are means  $\pm$  S.E.M.

of animals ( $n=4$ ) studied after 10 s of hypoxia, in an attempt to maintain  $P_{aO_2}$  as at 10 and 20 s of hypoxia. However the  $P_{aO_2}$  obtained was not significantly different from that observed with 8%  $O_2$ . For this reason, all values obtained at 10 s were pooled. Also the two control groups were pooled, there were 9 control animals, 10 hypoxic animals studied at 10 s, and another 6 studied at 20 s. The mean  $P_{aO_2}$  and  $P_{vO_2}$  values obtained are shown in Fig. 1. At 10 and 20 s, the mean arterial  $P_{aO_2}$  was 38.0 and 31.5 mm Hg, and  $P_{vO_2}$  was reduced by 8-10 mm Hg.

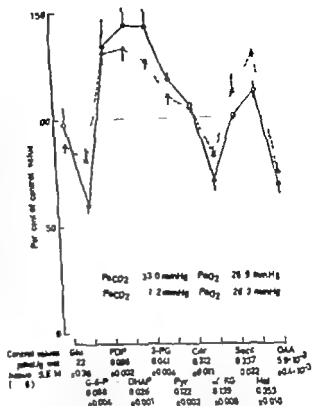
Fig. 2 shows the pattern of changes in glycolytic and citric acid cycle intermediates and 20 s of hypoxia as compared to the control values, the absolute values of which are given at the bottom. It should be noted that the control values obtained in unanesthetized animals after freeze-clamping were very close to those obtained with nitrous oxide anesthesia and surface freezing through the bone (see Norberg and Sjöbör 1975 a, and b, and c). Although the changes observed at 20 s were somewhat more marked than those seen at 10 s, the pattern was consistent. There were no changes in glycogen but significant reduction in glucose content. However this reduction should be interpreted with caution since, in any given animal, the glucose content in the tissue must depend on the blood glucose con-

TABLE I. Cerebral cortex concentrations of ATP, ADP, AMP, PCr, Cr and NH<sub>4</sub> in unanesthetized rats following administration of gases with low oxygen content (6-8%  $O_2$ ) for 10 or 20 s. The values expressed in  $\mu$ mol/g of wet tissue, are means  $\pm$  S.E.M.

Time of hypoxia	n	ATP	ADP	AMP	PCr	Cr	NH <sub>4</sub>
0	9	2.75 $\pm 0.04$	0.399 $\pm 0.012$	0.060 $\pm 0.003$	3.17 $\pm 0.11$	6.84 $\pm 0.16$	0.29 $\pm 0.011$
10 s	10	2.67 $\pm 0.05$	0.421 $\pm 0.015$	0.068 $\pm 0.007$	2.79 $\pm 0.07$	7.28 $\pm 0.26$	0.196 $\pm 0.022$
20 s	7	2.61 $\pm 0.03$	0.475 $\pm 0.018$	0.111 $\pm 0.012$	2.15 $\pm 0.09$	8.26 <sup>++</sup> $\pm 0.22$	0.27 <sup>++</sup> $\pm 0.027$



4. Influence of hypoxia (reduced arterial  $P_{O_2}$  to 26-27 Hg) upon cerebral cortex metabolism of glycolytic and acid cycle intermediates in anesthetized with nitrous oxide (70%). The values obtained in normocapnic animals ( $P_{aCO_2}$  38 mm Hg) were compared to previously obtained in isocapnic animals (Harburg and *et al.* 1975 and *et al.*). The values mean  $\pm$  S.E.M. in per cent of control, that are given at the top. Filled symbols denote values significantly different from controls ( $p < 0.05$ ). See also Fig. 2. For further abbreviations, see Fig. 2.



poxic animals the arterial  $P_{O_2}$  could be kept at  $41.2 \pm 0.7$  mm Hg, values that were slightly higher than those of the controls ( $37.9 \pm 0.4$  mm Hg). The difference ensured that tissue  $P_{O_2}$  was kept at, or just above, the normal value even if there was an increase in cerebral blood flow (see Bechgaard *et al.* 1964).

Fig. 4 shows the changes observed in glycolytic and citric acid cycle metabolites in nor-

mal II. Influence of reduction in arterial  $P_{O_2}$  to 26 mm Hg on tissue metabolites in normocapnic rats, anesthetized with nitrous oxide. The duration of hypoxia was 45 s. Values are means  $\pm$  S.E.M. ( $n = 6$  in both groups).

	Control	Hypoxia Normocapnic
Glu	$4.35 \pm 0.06$	$3.79 \pm 0.04^{***}$
TP	$6.04 \pm 0.03$	$6.36 \pm 0.10^{**}$
DP	$3.02 \pm 0.03$	$2.96 \pm 0.03$
MP	$0.299 \pm 0.006$	$0.304 \pm 0.005$
actate	$0.026 \pm 0.001$	$0.030 \pm 0.001$
act. Py	$1.32 \pm 0.03$	$1.38 \pm 0.07^{**}$
act. Py	$10.8 \pm 0.3$	$14.1 \pm 0.3^{***}$
actate	$13.4 \pm 0.2$	$13.8 \pm 0.1$
actate	$3.85 \pm 0.11$	$3.59 \pm 0.13$
act. OAA	$68 \pm 3$	$103 \pm 4^{***}$

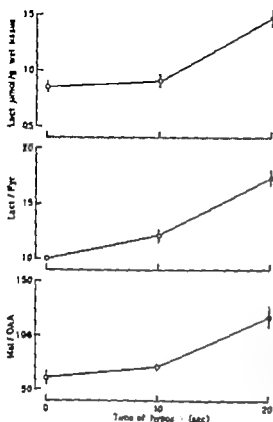


Fig. 3 Influence of hypoxia (administration of 0%  $\text{O}_2$ ) on cerebral cortex concentrations of lactate and on calculated lactate/pyruvate and malate/oxaloacetate ratios in unanesthetized animals.  $\pm$  S.E.M. Filled symbols denote values significantly different from the controls ( $p < 0.05$ ).

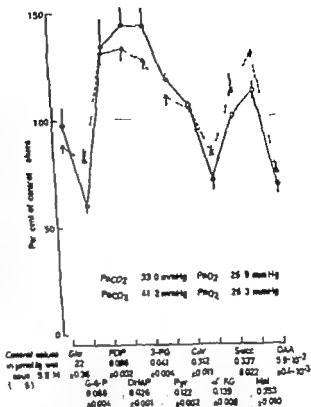
and malate/oxaloacetate ratios (Fig. 3). At 20 s, the lactate content and the ratios for redox couples were significantly increased.

Table I illustrates the tissue concentrations of ATP, ADP, AMP, PCr, creatine and  $\text{NH}_4^+$ . It should be noted that, although "freeze-clamping" introduces a very short delay between interruption of cerebral circulation and freezing of the tissue, the values for organic phosphates were less "optimal" than those obtained with surface freezing without previous interruption of circulation (see Norberg and Siesjö 1975a and below). Thus, PCr was about  $\mu\text{mol/g}$  lower, ADP about  $0.1 \mu\text{mol/g}$  higher and AMP about twice as high as with surface freezing. Although these differences indicate that there were some autolytic changes between control and hypoxic brains. Thus, at 20 s, there was a highly significant decrease in PCr and increase in Cr, there were significant increases in ADP and AMP and a significant reduction in ATP. The data obtained at 10 s indicated that smaller but similar changes occurred but only the decrease in PCr was statistically significant. The  $\text{NH}_4^+$  concentration was normal at 10 s of hypoxia but significantly increased at 20 s. The data allow a discussion of activation of glycolysis in terms of changes in concentration of glycolytic modulators (Discussion).

#### Anesthetized animals

In the hypoxic animals, the arterial  $\text{P}_{\text{O}_2}$  was reduced to  $26.3 \pm 0.6 \text{ mm Hg}$  as compared to  $144 \pm 6 \text{ mm Hg}$  in the controls. By addition of  $\text{CO}_2$  to the gas mixture administered to

4. Influence of hypoxia (reduction in arterial  $P_{O_2}$  to 26-27 Hg) upon cerebral cortex activities of glycolytic and acid cycle intermediates in anesthetized alb. rats (70%). The values obtained in normoxic animals ( $P_{aCO_2}$  33 mm Hg) were compared to previously obtained in accept animals (Norberg and *et al.* 1973 and *et al.*). The values mean  $\pm$  S.E.M. in per cent of value, that are given at the top. Filled symbols denote as significantly different from controls ( $p < 0.05$ ). See also *et al.* For further abbreviations, Fig. 2.



poor animals the arterial  $P_{O_2}$  could be kept at  $41.2 \pm 0.7$  mm Hg, values that were slightly higher than those of the controls ( $37.9 \pm 0.4$  mm Hg). The difference ensured that tissue  $P_{O_2}$  was kept at, or just above, the normal value even if there was an increase in cerebral blood flow (see Bachard *et al.* 1964).

Fig. 4 shows the changes observed in glycolytic and citric acid cycle metabolites in nor-

mal II Influence of reduction in arterial  $P_{O_2}$  to 26 mm Hg on tissue metabolites in normoxic rats, anesthetized with nitrous oxide. The duration of hypoxia was 45 s. Values are mean  $\pm$  S.E.M. ( $n = 6$  in both groups)

	Control	Hypoxia Normoxia
	4.35 $\pm$ 0.06	3.79 $\pm$ 0.04
	6.04 $\pm$ 0.03	6.36 $\pm$ 0.10**
TP	3.01 $\pm$ 0.03	2.96 $\pm$ 0.05
DP	0.299 $\pm$ 0.006	0.304 $\pm$ 0.005
MP	0.026 $\pm$ 0.001	0.030 $\pm$ 0.001
acetyl	1.32 $\pm$ 0.05	1.28 $\pm$ 0.07**
acetyl/Pyr	10.8 $\pm$ 0.3	14.1 $\pm$ 0.3**
Isocitrate	13.4 $\pm$ 0.2	13.8 $\pm$ 0.1
oxaloacetate	3.85 $\pm$ 0.11	3.79 $\pm$ 0.13
and GAA	60 $\pm$ 3	103 $\pm$ 4***

normocapnic animals (triangles) and compares them to the corresponding changes at an identical  $P_{O_2}$  in animals that became hypocapnic during the 45 s period of (circles, values taken from Norberg and Siesjö 1975 a). The values for F-6-P were since they varied unduly in the normocapnic group. The results demonstrate that the of changes observed in the normocapnic animals was very similar to that previously of in hypocapnic animals. Thus, activation of glycolysis at the PFK step seems to occur the tissue  $P_{CO_2}$  is held constant (see Discussion).

Table II shows the organic phosphates, the lactate contents, the lactate/pyruvate as well as the glutamate and aspartate concentrations. From the latter and from the concentrations (see Fig. 4), it was possible to derive OAA concentrations and, thereby, malate/OAA ratios (see Methods). There was a highly significant decrease in PCr, an increase in creatine, and a small rise in AMP but no significant change in ADP. The lactate/pyruvate and malate/OAA ratios were significantly increased.

### Discussion

Freezing techniques suitable for studying cerebral metabolic changes during short periods of hypoxia in unanesthetized animals include the "brain blower" developed by Locksley (1973) and the "freeze-clamping" method (Qubitorff 1975). Data obtained on control animals in the present series indicate that the techniques are comparable. Thus, although Locksley *et al* (1973) reported somewhat higher values for PCr the ADP concentration was higher and the AMP values were similar. However both these techniques give values for organic phosphates that are somewhat less "optimal" than those obtained by the simultaneous freezing technique of this laboratory (Pontén *et al* 1973). Probably these differences are due to the fact that, in freeze blowing or freeze-clamping, the circulation is interrupted before freezing occurs, and even if the delay before freezing is only 1-2 s, minor autolytic changes seem to occur. As remarked these changes are so small though, that they should not in any way invalidate the conclusions drawn from the present study. It should also be noted that freeze-clamping does not seem to affect the concentrations of glycolytic or citric acid cycle intermediates.

The present results, which extend those previously published (Norberg and Siesjö 1974 a and b), provide answers to the questions raised in the Introduction. The main results concern the activation of glycolysis, and the "initial" changes occurring in citric acid cycle intermediates and organic phosphates. The glycolytic changes observed can be interpreted according to the postulate of Krebs (1957) which states that when the flux through the pathway is increased there should be a decrease in the concentration of the substrate of a regulatory enzyme. In the previous studies it was not possible to prove that the flux was increased during hypoxia, but the present results show that this must be the case. Between 10 and 20 s of hypoxia the total sum of glycolytic intermediates plus lactate increased with  $0.57 \mu\text{mol/g}$  of which lactate formation accounted for  $0.55 \mu\text{mol/g}$ . The oxygen consumption of the cerebral cortex in Wistar rats is about  $4.5 \mu\text{mol/(g min)}$  (Norberg and Siesjö 1974) which corresponds to a glucose consumption of about  $0.8 \mu\text{mol/(g min)}$ . Thus, since the normal production of pyruvate should be about  $1.6 \mu\text{mol/(g min)}$ , or about  $0.25 \mu\text{mol/g}$  for a 10

mod, accumulation of  $0.55 \mu\text{mol/g}$  of lactate between 10 and 20 s shows that glycolysis was increased. Therefore, the glycolytic rate must have been at least doubled. These arguments could be wrong if pyruvate were formed in large amounts via the alanine aminotransferase reaction or from citric acid cycle intermediates in a reversal of  $\text{CO}_2$  fixation reactions. However this possibility can be excluded since hypoxia is accompanied by an increase in alanine concentration, and by an increase in the sum of citric acid cycle intermediates (Norberg and Siesjö 1975 b). Thus, in view of these arguments, and of the fact that signs of PFK activation occurred before pyruvate had accumulated, it can be concluded that activation of glycolysis was present.

In view of the fact that glycolytic rate was increased, and that there were decreases in PCr and F-6-P it can be concluded that PFK was activated. In the present unanesthetized male, this occurred already 10 s after the induction of hypoxia when the pyruvate and lactate concentrations had not yet increased. In the previous study (Norberg and Siesjö 1975 a) there were very small changes in organic phosphates, and it was concluded that if activation of PFK is secondary to a perturbation of the energy state, the enzyme must respond to very small changes in PCr, ADP and P. The present experiments confirm this conclusion since only PCr and creatine were significantly altered at 10 s.

The present results showed that changes in the concentrations of KG and OAA develop in parallel with those of glycolytic intermediates. At 10 and 20 s, malate was not significantly changed, and the increase in malate/OAA ratio was thus due to a fall in OAA concentration. The present findings give support to the previously proposed sequence of events during hypoxia (Duffy *et al.* 1972, Norberg and Siesjö 1975 a and b). Thus, it seems probable that increased malate/OAA ratio, by causing a relative reduction in OAA concentrations, is accompanied by a shift of the aspartate aminotransferase reaction towards glutamate formation, giving rise to reduction in the aspartate and  $\alpha$ -KG levels. Secondly this decrease in  $\alpha$ -KG can, together with a subsequent increase in pyruvate, also explain that there is initially a shift in the alanine aminotransferase reaction with a rise in alanine concentration. As judged from the changes in PCr, ATP, ADP and AMP (Table I), the perturbation of the energy state of the tissue in the present unanesthetized animals was larger than that observed previously on animals anesthetized with nitrous oxide (Norberg and Siesjö 1975 a). However the facts indicate that this is not necessarily the effect of anaesthesia. First, the present results pertain to only 10 and 20 s of hypoxia. There are results which suggest that a compensatory increase in cerebral blood flow (CBF) develops during the first 2 min of hypoxia (Longstrom *et al.* 1975). Thus, there may be a relatively large initial derangement of cerebral energy state which is later diminished due to hyperemia. Second, in preliminary experiments on the application of the present freezing technique, it was found that semi-steady state hypoxia ( $\text{P}_{\text{aO}_2}$  about 25 mm Hg for 30 min) was associated with a fall in PCr, but no detectable changes in ATP, ADP or AMP. These results are in accordance with previous observations obtained in  $\text{N}_2\text{O}$  anaesthesia (Bachelard *et al.* 1974). In view of these facts, we tentatively conclude that anaesthesia with  $\text{N}_2\text{O}$  does not significantly influence the response of the tissue to hypoxia.

The experiments carried out on anesthetized animals to evaluate the effect of  $\text{CO}_2$  tension demonstrate that, at 45 s, there are signs of activation of PFK even if tissue  $\text{P}_{\text{CO}_2}$  is held

normocapnic animals (triangles) and compares them to the corresponding changes of at an identical  $P_{O_2}$  in animals that became hypocapnic during the 45 s period of hypoxia (circles, values taken from Norberg and Sjöjög 1975 a). The values for F-6-P were constant since they varied unduly in the normocapnic group. The results demonstrate that the pattern of changes observed in the normocapnic animals was very similar to that previously observed in hypocapnic animals. Thus, activation of glycolysis at the PFK step seems to occur at the tissue  $P_{O_2}$  is held constant (see Discussion).

Table II shows the organic phosphates, the lactate contents, the lactate/pyruvate ratio as well as the glutamate and aspartate concentrations. From the latter and from the malate/OAA ratios (see Fig. 4) it was possible to derive OAA concentrations and, therefore, malate/OAA ratios (see Methods). There was a highly significant decrease in PCr, an increase in creatine, and a small rise in AMP but no significant change in ADP. The lactate/pyruvate and malate/OAA ratios were significantly increased.

### Discussion

Freezing techniques suitable for studying cerebral metabolic changes during short periods of hypoxia in unanesthetized animals include the "brain blower" developed by Veatch *et al.* (1973), and the "freeze-clamping" method (Quistorff 1975). Data obtained on control animals in the present series indicate that the techniques are comparable. Thus, although Veatch *et al.* (1973) reported somewhat higher values for PCr, the ADP concentration was also higher and the AMP values were similar. However both these techniques give values for organic phosphates that are somewhat less "optimal" than those obtained by the slow freezing technique of this laboratory (Pontén *et al.* 1973). Probably these differences are due to the fact that, in freeze blowing or freeze-clamping, the circulation is interrupted before freezing occurs, and even if the delay before freezing is only 1-2 s, minor autolytic changes seem to occur. As remarked, these changes are so small, though, that they should not in any way invalidate the conclusions drawn from the present study. It should also be noted that freeze-clamping does not seem to affect the concentrations of glycolytic or citric acid cycle intermediates.

The present results, which extend those previously published (Norberg and Sjöjög 1974 a and b) provide answers to the questions raised in the introduction. The main results concern the activation of glycolysis, and the "initial" changes occurring in citric acid cycle intermediates and organic phosphates. The glycolytic changes observed can be interpreted according to the postulate of Krebs (1957) which states that when the flux through the pathway is increased, there should be a decrease in the concentration of the substrate of a regulatory enzyme. In the previous studies it was not possible to prove that the flux was increased during hypoxia, but the present results show that this must be the case. Between 10 and 20 s of hypoxia the total sum of glycolytic intermediates plus lactate increased with 0.57  $\mu\text{mol/g}$  of which lactate formation accounted for 0.55  $\mu\text{mol/g}$ . The oxygen consumption of the cerebral cortex in Wistar rats is about 4.5  $\mu\text{mol/(g min)}$  (Norberg and Sjöjög 1974) which corresponds to a glucose consumption of about 0.8  $\mu\text{mol/(g min)}$ . Thus, since the normal production of pyruvate should be about 1.6  $\mu\text{mol/(g min)}$ , or about 0.25  $\mu\text{mol/g}$  for a 10 s

## Effects of Histamine, Vasopressin, and Angiotensin II on Hepatosplanchnic Hemodynamics, Liver Function, and Hepatic Metabolism in Cats

By

N. KRARUP

Received 21 April 1975

### Abstract

**ABSTRACT** *N* Effects of histamine, vasopressin, and angiotensin II on hepatosplanchnic hemodynamics, liver function and hepatic metabolism in cats. *Acta physiol. scand.* 1975. 95: 311-312.

Removes infusions of histamine (5 µg/kg min), vasopressin (10 mU/kg min), or angiotensin II (5 µg/kg min) were given to fasting cats. Hepatic arterial flow was decreased 30% by histamine, increased 4% by vasopressin, and not significantly affected by angiotensin. Portal venous flow was increased 33% by histamine, decreased 40% by vasopressin, and not significantly affected by angiotensin. Hepatic arterial conductance was decreased about 25% by histamine and angiotensin, and not significantly affected by vasopressin. The gastrointestinal conductance was decreased about 40% by vasopressin and angiotensin, and increased 25% by histamine. The conductance in the intrahepatic low pressure vessels was not affected by histamine and vasopressin, but decreased 25% during the infusion of angiotensin. On hemodynamic effects, however, are not accompanied by changes in the liver function or hepatic metabolism as judged from the splanchnic elimination of ethanol, the hepatic uptake and secretion of HCG, hepatic oxygen consumption, and lactate and ketone production. This indicates that the functional capacity of the liver and thereby the number of sinusoids perfused is not markedly influenced by these drugs. Vasopressin caused decrease in the oxygen consumption and an increase in the lactate production in the portal splanchnic area, both may be due to redistribution of the gastrointestinal blood flow.

Despite numerous studies on the hepatosplanchnic circulatory response to vasoactive substances possible relationships between drug effects on vascular functional and metabolic parameters in the splanchnic area has only been sparsely investigated (Greenway and Lark 1971).

In previous experiments the effects of catecholamines, glucagon and dibutyryl-cyclic AMP on the splanchnic vascular system were found to be independent of effects on the hepatic metabolism and liver function (Krarup 1973, Krarup and Larsen 1974, Krarup, Arnes and Munch 1975).

In the present experiments the effects of continuous i.v. infusions of histamine, vasopressin and angiotensin II on hepatosplanchnic hemodynamics were tested together with a simultaneous investigation of the liver function and splanchnic metabolism.

Liver function was judged from the splanchnic elimination of ethanol, the secretion of

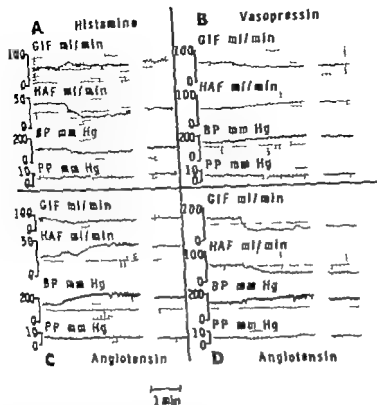
constant. There is thus no principal difference in response between normocapnic and capnic animals. In all probability the pattern typical of PFK activation disappears rapidly in normocapnic animals because the developing tissue acidosis retards the of PFK, relative to that of other regulatory enzymes.

This study was supported by grants from the Swedish Medical Research Council (Project No and 14X 2179), from the Swedish Tercentenary Fund, from US PHS Grant No RO1 NS0 31 NIH from the Swedish Board for Technical Development.

## References

- BACUZARD H. S., L. H. LEWIS, U. PORTÉN and B. K. SUND, Mechanisms activating glycine brain in arterial hypoxia. *J Neurochem.* 1974. 22. 395-401.
- BORGSTRÖM, L., H. JÖHANSSON and B. K. SUND, The relationship between arterial  $P_{O_2}$  and cerebral flow in hypoxic hypoxia. *Acta physiol. scand.* 1975. 93. 423-432.
- DELCHER, H. K. and J. C. SHUFF, Effect of pH,  $pCO_2$  and bicarbonate on metabolism of glucose in rat heart. *Biochim. biophys. Acta (Amst.)* 1966. 121. 250-260.
- DUFFY, T. E., S. R. NELSON and O. H. LOWRY, Cerebral carbohydrate metabolism during acid and recovery. *J Neurochem.* 1972. 19. 959-977.
- FOLKSGROVÁ, J., B. LJUNGBERGMAN, K. NORBERG and B. K. SUND, Influence of complete ischemic metabolic metabolites, citric acid cycle intermediates, and associated amino acids in the rat cerebellum. *Brain Res.* 1974. 80. 265-279.
- KREBS, H. A., Equilibrium in transamination systems. *Biochem. J.* 1933. 54. 8-26.
- KREBS, H. A., Control of metabolic processes. *Endocrine* 1957. 16. 125-132.
- LOWRY, O. H. and J. V. PASSONNEAU, The relationship between substrates and coenzymes of glycolysis in brain. *J Biol. Chem.* 1964. 239. 31-42.
- LOWRY, O. H. and J. V. PASSONNEAU, Kinetic evidence for multiple binding sites on phosphoenolpyruvate. *J Biol. Chem.* 1966. 241. 2268-2279.
- LOWRY, O. H. and J. V. PASSONNEAU, *A Flexible System of Enzymatic Analysis*. Academic Press, New York and London 1972.
- NORBERG, K. and B. K. SUND, Metabolism of oxygen, glucose, lactate and pyruvate in the rat brain. *J Neurochem.* 1974. 22. 1127-1129.
- NORBERG, K. and B. K. SUND, Cerebral metabolism in hypoxic hypoxia. I. Pattern of activation. *Brain Res.* 1975 a. 86. 31-44.
- NORBERG, K. and B. K. SUND, Cerebral metabolism in hypoxic hypoxia. II. Citric acid cycle intermediates and associated amino acids. *Brain Res.* 1975 b. 86. 45-54.
- PORTÉN, U., R. A. RATCHESON, L. O. SALFORD and B. K. SUND, Optimal freezing conditions for metabolites in rats. *J Neurochem.* 1973. 21. 1177-1178.
- QUISTORFF, B., Mechanical device for rapid removal and freezing of liver or brain tissue from anesthetized and nonparalyzed rats. *Analyt. Biochem.* 1975. 1. press.
- VEATCH, R. L., R. L. HARRIS, D. VELHO and E. H. VEATCH, Freeze-blowing: a new technique for study of brain in vivo. *J Neurochem.* 1973. 20. 183-188.





1. The effects of conscious infusions of histamine, vasopressin, and angiotensin on gastrointestinal portal venous flow (GIF), hepatic arterial flow (HAF), mean arterial blood pressure (BP), and portal venous pressure (PP). The drug infusions start at the beginning of the curves, and the last part of the curves is steady state levels during the drug infusion.

et (Fig. 1 A). This was accompanied by a more pronounced decrease in hepatic arterial flow (from  $15 \pm 1$  ml/kg min to  $11 \pm 1$  ml/kg min when steady state in the infusion was attained). Despite the decrease in arterial blood pressure, portal venous flow rose (from  $15 \pm 4$  to  $21 \pm 4$  ml/kg min). EHBF averaged  $31 \pm 2$  ml/kg min in the control period, and during histamine infusion a value of  $32 \pm 2$  ml/kg min was found. Portal venous pressure varied nearly in parallel with the portal venous flow. The hepatic arterial conductance decreased, gastrointestinal conductance increased and portal venous conductance remained nearly unaltered (Fig. 2).

2. *Vasopressin.* The infusion of vasopressin  $10$  mU/kg min caused a rise in arterial blood pressure which within a few minutes levelled off about 20% above control pressure (Fig. 1 B). Hepatic arterial flow increased slightly more than arterial blood pressure (from  $12 \pm 1$  to  $16 \pm 2$  ml/kg min). Despite the increase in arterial blood pressure portal venous flow increased (from  $11 \pm 1$  to  $11 \pm 1$  ml/kg min). In the control period EHBF averaged  $33 \pm 4$ , and during vasopressin infusion a value of  $29 \pm 4$  was found. Portal venous pressure varied

bile and the hepatic uptake and excretion of Indocyanine Green (ICG). The metabolism was followed by determinations of the oxygen consumption and output of lactate, pyruvate, ketone bodies and glucose in the splanchnic area.

### Methods

15 fasting cats, anesthetized with chloralose (50 mg/kg) and an initial dose of Nembutal (30 mg/kg) used for the experiments. The methods employed were as previous described (Krarup 1973a). (For infusion, blood sampling and pressure recordings were placed in the femoral vein and a hepatic vein and the portal vein. Electromagnetic flowprobes were placed around the portal vein and a common hepatic artery after ligation of the right gastroduodenal artery. Bile was collected from the choledochus after ligation of the cystic duct.

At the end of the operation priming doses of ethanol (6.5 mmol/kg) and ICG (300  $\mu$ g/kg) was injected into a femoral vein, followed by continuous infusions of ethanol (36  $\mu$ mol/kg min), ICG (5  $\mu$ g/kg min) and taurocholate (0.2  $\mu$ mol/kg/min). After a recovery and equilibration period of 90 minutes blood samples were drawn from a femoral artery and a hepatic vein at 15 min intervals for determination of the concentrations of ethanol, ICG, lactate (L), pyruvate (P),  $\beta$ -hydroxybutyric acid (HB), acetoneacetate (Ac), and oxygen. Samples from the portal vein for determination of oxygen saturations and—when vasopressin experiments—lactate were also drawn. Bile samples were collected for determination of ICG flow and ICG and electrolyte concentrations. Following a 60 min control period, histamine (5  $\mu$ g/kg min) or angiotensin (0.5  $\mu$ g/kg min) were given intravenously to groups of cats, and the parameters were followed in another 60 min period.

#### Calculations

The splanchnic uptake or output of the different substances were calculated from the arterio-venous concentration differences and the estimated hepatic blood flow (EHBF) determined by measuring ICG using the Fick principle. The gastrointestinal—including the splenic—oxygen consumption and output were calculated from the portal venous flow and the arterio-portal gradients of oxygen and blood. The hepatic oxygen consumption was calculated as the difference between the splanchnic and the gastrointestinal oxygen consumption. Clearance and extraction ratio of ICG were calculated by dividing the amount of ICG eliminated and the arterio-hepatic venous ICG difference by the arterial plasma concentration of ICG. The vascular conductances in the gastrointestinal area (GIC), the hepatic artery (HAC) and the intrahepatic low pressure vessels (PVC) were calculated as

$$GIC = \frac{GIF}{BP - PP} \text{ ml/kg min} \times \text{mm Hg}^{-1}$$

$$HAC = \frac{HAF}{BP}$$

$$PVC = \frac{GIF}{PP}$$

(GIF gastrointestinal (= portal venous) flow; HAF hepatic arterial flow; BP= mean arterial blood pressure; PP= portal venous pressure). In accordance with previous findings (Krarup 1973b) hepatic venous pressure was not statistically different from zero. As, in a preliminary series, neither of the drugs were found to influence hepatic venous pressure, this was set as zero in the calculations.

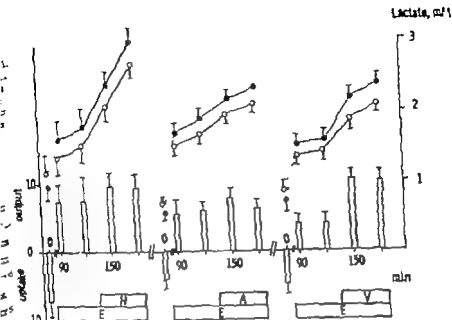
#### Statistical procedures

The effects of the drugs were tested by Student's *t* test for paired comparisons.

### Results

#### Hepatosplanchnic hemodynamics.

1 *Histamine* The infusion of histamine 5  $\mu$ g/kg min caused an immediate decrease in arterial blood pressure, which in the course of few minutes returned towards the control



1. The concentrations of lactate in arterial (O) and hepatic venous (●) blood, and the splanchnic output of lactate. The bars indicate S.E., E ethanol infusion, H, A, and V the infusions of histamine, angiotensin, and vasopressin.

Steady arterial lactate concentrations were not obtained in the control periods, but as is seen from Fig. 3, histamine and vasopressin, in contrast to angiotensin, caused a further rise in the lactate concentrations. Histamine and angiotensin did not affect the splanchnic lactate output significantly whereas the infusion of vasopressin increased the splanchnic lactate output by 100%. In two of the vasopressin experiments portal venous lactate concentrations were measured. The gastrointestinal lactate output which was insignificant in the control period increased to about  $6 \mu\text{mol/kg min}$  when vasopressin was given. This accounted fully for the increase in splanchnic lactate output, thus the hepatic production of lactate was not affected by vasopressin. Before as well as during the drug infusions the portal venous pyruvate concentrations were so small that the L/P ratio in hepatic venous blood could not be calculated with sufficient accuracy. There was a splanchnic uptake of pyruvate of about  $1.0 \mu\text{mol/kg min}$  before as well as during the drug infusions. Infusion of the vasoactive drugs had no significant effects on the splanchnic production of ketone bodies and neither was the hepatic venous HB/Ac ratio affected. Histamine infusion was followed by an immediate, pronounced (from 15 to  $35 \mu\text{mol/kg min}$ ) but shortlasting (15 min) increase in the splanchnic glucose output, whereas neither vasopressin nor angiotensin had measurable effects on the glucose concentration and splanchnic glucose output.

#### Discussion

The present hemodynamic effects of the drugs agree with previous findings (for review see Atterway and Stark 1971), except for the constriction in the hepatic arterial bed observed

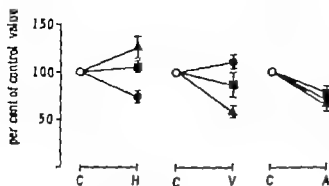


Fig. 2. The vascular conductance in the gastrointestinal area (A), the hepatic artery (H), and in the hepatic pressure vessels (V) during the infusion of histamine (H), vasopressin (V) or angiotensin (A), expressed as per cent of the values in the control period. The bars indicate S.E.

nearly in parallel to the portal venous flow. The hepatic arterial and portal venous pressures were not significantly affected and the gastrointestinal conductance decreased.

**3. Angiotensin** The infusion of angiotensin  $0.5 \mu\text{g/kg min}$  caused an immediate rise in arterial blood pressure, initially accompanied by cardiac arrhythmia. In the course of 5 min the arrhythmia disappeared, and the arterial blood pressure levelled off about 6% above control level. In 4 of the cats hepatic arterial flow increased, somewhat less than the arterial blood pressure, and portal venous flow and pressure either remained constant or were slightly decreased (Fig. 1 C). In the last cat with a small pressure response to angiotensin, hepatic arterial as well as portal venous flows were markedly reduced, whereas an increase in portal pressure was observed (Fig. 1 D). For the whole group hepatic arterial flow, portal venous flow and portal pressure in the control period averaged  $18 \pm 2$ ,  $14 \pm 1$  (ml/kg min) and  $58 \pm 0.5$  (mm Hg) respectively. During angiotensin infusion hepatic arterial flow averaged  $20 \pm 3$  ml/kg min, portal flow  $16 \pm 1$  ml/kg min and portal pressure  $70 \pm 0.6$  mm Hg. EHBP in the control period averaged  $32 \pm 4$  ml/kg min and was not significantly changed by angiotensin ( $34 \pm 4$  ml/kg min). Both the hepatic arterial, gastrointestinal and the portal venous conductance decreased (Fig. 2).

#### Liver function

None of the drugs influenced the splanchnic elimination rate of ethanol. Histamine and angiotensin had no effect on the plasma clearance and extraction ratio of ICG, whereas vasopressin caused a 10 per cent decrease in ICG clearance ( $p < 0.01$ ) without significant effect on the extraction ratio of the dye. The biliary recovery of ICG was nearly complete (95%) and not changed by the drug infusions. All drugs caused a 10 per cent decrease in bile flow. The biliary concentrations of sodium, potassium, chloride, and bicarbonate were not affected.

#### Splanchnic metabolism

Histamine and angiotensin had no effect on the gastrointestinal and hepatic oxygen consumption. During infusion of vasopressin the gastrointestinal oxygen extraction increased (portal venous oxygen saturation percent was  $56 \pm 9$  in the control period,  $46 \pm 4$  during vasopressin infusions) but not enough to compensate for the decrease in gastrointestinal flow so that the gastrointestinal oxygen consumption decreased (from  $47 \pm 5$  to  $33 \pm 4$   $\mu\text{mol/kg min}$ ). The hepatic oxygen consumption was not affected by vasopressin.

obic glycolysis as surmised from the decrease in gastrointestinal oxygen consumption. It is probably not a generalized hypoxia in the gastrointestinal area, as the oxygen saturation of the portal venous blood was only moderately decreased, but may be due to redistribution of the gastrointestinal flow from nutritive vessels to arterio-venous shunts. Histamine apparently stimulates hepatic glycogenolysis, whereas angiotensin and vasopressin have no such effect, the latter in contrast to findings in perfused rat livers (Hems and White 1973).

### Conclusions

Administration of histamine, vasopressin and angiotensin at doses producing changes in the hepatoportal hemodynamics has no significant effect on liver function as judged from the elimination rate of ethanol and the hepatic uptake and excretion of ICG whereas a slight decrease in the production of ductular bile may result. Nor do the drugs affect the hepatic oxygen consumption or lactate production. This indicates that the number of perfused sinusoids in the liver is not changed by the drugs, and that redistribution of the intrahepatic blood flow leading to localized hypoxia is not elicited. Vasopressin may cause a redistribution of gastro-intestinal flow from nutritive channels to arteriovenous shunts.

Skilled technical assistance of Mrs J. Justesen and Mrs L. B. Nielsen is gratefully acknowledged.

### References

1. M. M. D. S. SCAR, J. R. McNALL and C. V. GAZDAR: Vasoconstrictor effects of vasopressin on resistance vessels of spleen, intestine and liver. *Amer. J. Physiol.* 1970, **218**, 1704-06.
2. C. V. GAZDAR and R. S. ARK: Hepatic vascular bed. *Physiol. Rev.* 1971, **51**, 23-65.
3. D. A. and P. D. WHITTON: Stimulation by vasopressin of glycogen breakdown and gluconeogenesis in the perfused rat liver. *Biochem. J.* 1973, **136**, 705-09.
4. H. E. R. L. RANCK and H. TYOSTRUP: Liver function and blood flow in normal rats during infusion of vasopressin. *Scand. J. Clin. Lab. Invest.* 1969, **24**, 279-84.
5. U. N. The effect of noradrenaline and adrenaline on hepatoportal hemodynamics, functional capacity of the liver and hepatic metabolism. *Acta physiol. scand.* 1973, **87**, 307-19.
6. U. N. The effect of hemorrhage on hepatoportal hemodynamics, liver function and hepatic metabolism. *Acta physiol. scand.* 1973, **89**, 260-77.
7. U. N. and J. A. LARSEN: The effect of glucagon on hepatoportal hemodynamics, functional capacity and metabolism of the liver in cats. *Acta physiol. scand.* 1974, **91**, 42-52.
8. U. N., J. A. LARSEN and A. MUNCK: Regulation of glucagon effects on splanchnic hemodynamics and liver function by N<sup>6</sup>, 2'-O-dibutyryl-3', 5'-cyclic AMP (DBcAMP) in cats. *Acta physiol. scand.* 1975, **95**, 110-116.
9. J. U. PRITER and E. BAUMHAGEN: Appearance and role of vasoconstrictor reactions in the liver regulation. *Pflügers Arch. ges. physiol.* 1968, **299**, 311-15.
10. U. N. and E. P. FICK: Über den durch "Schockgifte" erzeugten Zirkulationsstörungen. *Monatsh. Med. Natur.* 1915, **34**, 1141-43.
11. K. WOLFE and T. SCHLIMM: Die Wirkung von Acetylcholin, Bradykinin und Angiotensin auf die Durchblutung der Leber des marktorientierten Hundes und auf den endständigen Druck in Ductus choledochus. *Pflügers Arch. ges. physiol.* 1968, **300**, 189-201.
12. R. SCHLIMM: Untersuchungen zur Reduktion der Leberfunktion bei der hämoglobinfrei perfundierten Ratte. In: *Effizienz der Leber bei der perfundierten Leber*. Ed. Starb, W. and R. Scholz, Springer Verlag Berlin-Heidelberg, 1968, p. 24-47.
13. A. Y. J. R. C. MORRIS and A. J. WATTS: Vasopressin effect on portal and systemic hemodynamics. *JAMA* 1960, **216**, 1065-68.
14. H. REILLY, K. S. KIDDO and N. TYOSTRUP: Clearance as a quantitative measure of liver function. In: *The Liver: quantitative aspects of structure and function*. Ed. Farnagartner, G., Karger Basel, 1973.

during histamine administration. This cannot be explained by a baroreceptor response to the decreased arterial blood pressure, as the hepatic nerves around the hepatic artery and portal vein had been cut to secure contact between the flow in the vessels. The constriction may be explained by a direct effect of histamine on the arterioles. Also, an increase in sinusoidal pressure—reflected by the increase in pressure—may elicit constriction of the hepatic arterioles (Lutz, Peiper and Ill 1968). The lack of effect of histamine on the conductance in the intrahepatic low-pressure vessels indicates that a histamine-sensitive hepatic venous sphincter does not exist in contrast to dogs (Mautner and Pick 1915). The decrease in gastrointestinal conductance during vasopressin infusion is a well documented finding and the resultant decrease in pressure has been used in the treatment of hemorrhage from oesophageal varices. A possible direct constrictor effect of vasopressin on the hepatic arterioles may be masked by a decrease as a response to the decrease in sinusoidal pressure, in accordance with the findings of Cohen *et al* (1970). In the study of Cohen *et al* (1970) the constrictor effect of angiotensin on the hepatic artery was less pronounced than in the present experiments. This discrepancy may be due to the relatively unaffected portal—and hence sinusoidal—pressure in the present study. The decreased portal venous conductance during angiotensin infusion is probably due to constriction of the intra-hepatic portal ramifications (Scholtz and Sørensen 1968).

According to the model for ICG elimination proposed by Winkler, Keiding and Tygstrup (1973) the decrease in ICG clearance during the infusions of vasopressin may be explained by the decrease in EHBP with an unaltered function of the ICG eliminating system. In other experiments where EHBP was not significantly changed, no effect on the ICG clearance was found. The small decrease in bile flow is probably due to a depression of ductular bile formation, since the excretion of ICG was unaffected and the excretion of bicarbonate decreased in proportion to the bile flow. Judged from the ethanol elimination, liver function was unaffected despite the significant changes in the distribution of hepatic blood flow on the portal vein and hepatic artery and/or the changes in hepatic arterial and portal venous conductances. The maintained functional capacity of the liver indicates that the number of functioning liver cells and thereby the number of sinusoids perfused was not affected by the drugs. For vasopressin the results agree with findings in humans, where infusion of vasopressin did not change the galactose elimination rate, a parameter reflecting the "functional liver mass" (Jacobsen, Ranek and Tygstrup 1964).

The unaltered hepatic oxygen consumption also reflects that gross uneven perfusion of sinusoids is not elicited by the drug infusions. The increase in the lactate concentration during infusion of histamine must be due to peripheral glycolysis, as the splanchnic lactate output was not affected. Neither did angiotensin influence the splanchnic lactate output. During the vasopressin infusions, a rise in the splanchnic lactate output was due to lactate production in the prehepatic splanchnic area. Hypoxia in even minor parts of the splanchnic venous blood (reflecting the mitochondrial redox level (Scholz 1968)), but such alterations were not observed. The gastro-intestinal lactate production during vasopressin infusion corresponds to the findings of Silva, Moffat and Watt (1968) and may be explained

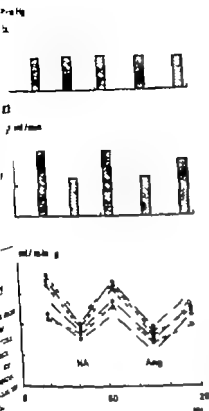


Fig. 1 Effect of norepinephrine (NA) and angiotensin (Ang) infusion on arterial pressure (AP), total renal blood flow (RBF) and local cortical blood flow (CBF) at 6 different electrode sites in one dog. Open circles: inner cortex. Closed circles: Outer cortex.

## Methods

Effect of adrenaline (A), norepinephrine (NA), angiotensin (Ang) (Hypertensin, CIBA) and vasopressin (V) (Pitressin, Parke, Davis & Co.) on renal blood flow was determined in 9 expts. on 6 mongrel dogs both sexes, weighing 15–21 kg. The animals had free access to water and food until 12 h prior to the experiment. The food consisted of fish and pellets providing about 3 g NaCl/24 h. Anaesthesia was induced i.v. injection of Nembutal, 25 mg/kg b.wt. Additional doses of 3 mg/kg were given when needed. Polyethylene catheters were placed in brachial vein, brachial and saphenous artery for infusions and arterial pressures (AP) recording. The kidney was exposed retroperitoneally through flank incision. An electromagnetic flowmeter probe (Pitot) was placed on the renal artery for continuous measurement of total renal blood flow (RBF). Calibration of the flowmeter was performed on the femoral artery. A polyvinyl catheter was inserted in the renal artery with the tip directed upstream. A Bialock clamp was placed on the aorta above the renal artery to control the renal arterial pressures during the infusion of vasoconstrictor agents. Three L-shaped platinum electrodes were placed in the outer cortex with the sensitive electrode tips 4 mm deep, and 3 other electrodes 5–8 mm deep in the inner cortex. The shafts of the electrodes were tied to the renal capsule by 2 sutures and the electrodes carefully covered with perirenal fat and the wound closed by towel clips. Hydrogen concentration in the tissue around the sensitive electrode tip was determined polarographically by polarisation potential of +0.1 V versus an Ag/AgCl electrode placed subcutaneously on the hip (Lelzard *et al.* 1973). The amplified electrode current was recorded on 6-channel recorder (Rüchardt) (Rüchardt Co., Model B-44). 3–10 ml saline saturated with hydrogen at 37°C was injected in the renal artery

## Effect of Vasoactive Agents on the Distribution of Renal Cortical Blood Flow in Dogs

By

I. TYRSEBOTH and A. KIRKEBO

Received 2 May 1975

---

### Abstract

TYRSEBOTH, I. and A. KIRKEBO. *Effect of vasoactive agents on the distribution of renal blood flow in dogs* Acta physiol. scand. 1975. 95. 318-328

The distribution of renal cortical blood flow was studied in 6 Nembutal anesthetized dogs during periods and during infusions of adrenaline, noradrenaline, angiotensin and vasopressin. Local blood flow was measured as  $H_2$  gas desaturation rate recorded polarographically by platinum electrodes in outer and inner cortex. The total renal blood flow (RBF) was measured by an electromagnetic meter. In the control period the outer cortical blood flow (OCF) and inner cortical blood flow (ICF) averaged  $3.59 (\pm S.D. 0.85)$  ml/min/g and  $3.23 (\pm S.D. 0.64)$  ml/min/g, respectively. Infusions of the vasoactive agents caused essentially equal vascular responses. All agents caused increased local resistance and reduction of RBF whether given intravenously or intraarterially. The RBF could be to 20-50 % of initial control flow by increasing doses of vasoactive agents. OCF and ICF fell proportionally and almost to the same extent as RBF or OCF fell slightly more than ICF. There was no patchy or zonal hypoperfusion in cortex caused by infusion of adrenaline, noradrenaline, angiotensin or vasopressin.

---

It is well known that adrenaline, noradrenaline, angiotensin and vasopressin increase vascular resistance in the kidney and decrease total renal blood flow (RBF). However, several investigators have claimed that the resistance is not increased in all parts of the kidney. Thus Trueta *et al* (1947) found in rabbits that large doses of adrenaline and angiotensin reduced the amount of Indian ink and angiographic contrast medium reaching the outer cortex, whereas more contrast was found in the juxtamedullary circulation. Recently Carriere (1969) and Grandchamp, Ayer and Truniger (1971), using external washout of  $^{133}Xe$  or  $^{86}Kr$ , reported that vasopressor infusion caused a marked reduction of flow in outer cortex, whereas flow in the juxtamedullary cortex and outer medulla remained unchanged or even increased. However, this evidence of a preferential vasoconstriction in the outer cortex has not been confirmed by measurements of microsphere distribution (Lund *et al* 1972) and local heat clearance (Gröningsjö and Persson 1971). In view of these conflicting results we have reinvestigated the question of the distribution of blood flow in the kidney during the renal vasoconstriction produced by several vasoactive agents, using the washout method which permits repeatable local blood flow measurements.



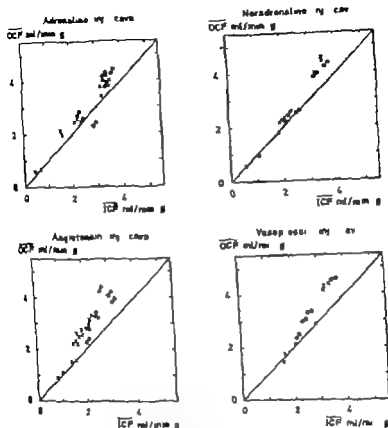


Fig. 2. Mean blood flow in outer cortex (OCF) related to mean flow in inner cortex (ICF) during infusion of the vasoconstrictor agents. Closed circles: Control period. Crosses: Test period.

1) ml/min g. Mean arterial pressure in control periods averaged 121 (range 100-145) mm Hg.

CBF and local blood flow fell progressively with increasing doses of all vasoconstrictor agents.

There was a proportional reduction of OCF and ICF or a slightly greater fall in OCF to ICF for each of the four vasoconstrictor agents: A, NA, Ang and ADH when infused to the renal artery (Fig. 2-6). The ratio  $OCF/ICF$  between mean flow of the three electrodes in outer cortex (OCF) to mean flow of the three electrodes in inner cortex (ICF) in different experiments was 1.05-1.23 in control (not significantly higher than unity), and fell to 0.92-1.14 during infusion of the four vasoconstrictor agents (Table I). The small decreases in the  $OCF/ICF$  ratio, 5-13% below control, were statistically significant for i.a. infusion of NA and Ang.

For all agents the mean ratio  $OCF/ICF$  calculated from local flow values in per cent of control, were insignificantly lower for flow values smaller than 67% than for flow values between 67-100% of control.

TABLE I. OCF/ICF ratio (S.E.) in control periods and during infusion of adrenaline (A), NA, angiotensin (Ang) and vasopressin (ADH).

Agents	Control	Arterial infusion	p, paired t test	Control	Venous infusion	n
A	1.18 ± 0.09	1.07 ± 0.05	< 0.05	1.10 ± 0.06	1.05 ± 0.05	
n	29	34		12	17	
NA	1.05 ± 0.07	0.92 ± 0.06	ns	1.10 ± 0.03	1.09 ± 0.04	n
n	20	29		10	15	
Ang	1.13 ± 0.07	1.14 ± 0.03	< 0.05	1.24 ± 0.04	1.29 ± 0.05	n
n	8	16		10	20	
ADH	1.14 ± 0.07	1.09 ± 0.06	ns	1.26 ± 0.04	1.13 ± 0.06	n
n	8	10		10	12	

p, calculated by paired t-test pairing one or two test ratios to the last control ratio measured before infusion periods. (All differences were nonsignificant using unpaired t-test.)

n is the number of infusion periods.

ns, nonsignificant.

through the polyvinyl catheter until constant  $H_2$ -concentration was obtained at the afferent sites. The injection was then suddenly stopped and the washout curves were recorded, and plotted on semilogarithmic paper. Blood flow was determined from the formula:  $0.69/T_{1/2}$ , where  $T_{1/2}$  is the half life of the hydrogen concentration in the tissue.

The vasoactive agents were dissolved in 0.9% saline and infused at a rate of 0.1–2.5 ml/min artery by a syringe pump (Sage Instrument, Mod. 355) or L. by a peristaltic pump (Miles) 0.2–15 ml/min. The concentration of A, NA, Ang and ADH in the infused solution were 2, 10, 10 µg/ml and 0.1 PU/ml respectively.

The local flow was determined at several infusion rates, reducing blood flow to all levels in and the lowest RBF which we could keep stable within 3% of control flow. Usually this level was 10–25% of control. The determination of the hydrogen washout rate as a constant RBF obtained by careful adjustment of the infusion rate of vasoactive agents in the Under LV infusion of large doses of vasoactive agents a rise in renal arterial pressure was lowered to 50–70 mm Hg by the Blalock clamp on the aorta. In all infusion periods the perfused

At the end of each experiment the kidney was excised, drained and weighed. The location of tips in cortex were carefully examined. Using Student paired t test, differences were considered at  $p < 0.05$ .

## Results

Infusions were usually sustained for 3 to 15 min before flow measurements. Reactions of different vasoactive agents were given with intervals of recovery before infusion as shown in Fig. 1. New infusion of a vasopressor agent was not until RBF and local cortical flow had increased close to control level.

*Renal arterial infusion of vasopressor agents* Control measurements of RBF determined by electromagnetic flowmeter varied between 3.20 and 6.40 ml/min/g kidney weight in dogs with an average of  $4.20 (\pm S.D. 1.20)$  ml/min/g. The local flow measured in cortex of individual dogs ranged from 2.17 to 4.64 ml/min/g with an average of  $3.0 \pm 0.85$  ml/min/g and flow in inner cortex from 2.33 to 4.23 with an average of  $3.0 \pm 0.85$  ml/min/g.

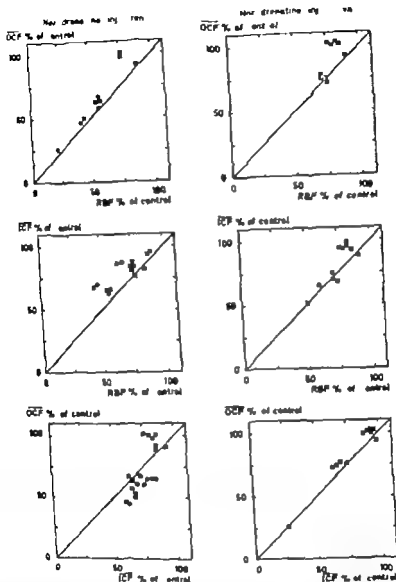


Fig. 4. The effect of noradrenaline infused into the renal artery and the vein on the total renal blood flow (RBF) and the local cortical blood flow (OCF/ICF). Open circles. Renal arterial pressure lowered and series clamped.

OCF) is plotted against the mean flow of 3 electrodes in the inner cortex (ICF) in the different experiments.

The OCF/ICF ratios in control measurements and during i.v. infusions of NA, Ang and ADH were 1.10 to 1.26, all significantly higher than unity. No significant change of the ratio was obtained during administration of NA, Ang and ADH (Table I). I.v. infusion of A caused a small, but statistically significant reduction in ratio, as did I.a. infusion.

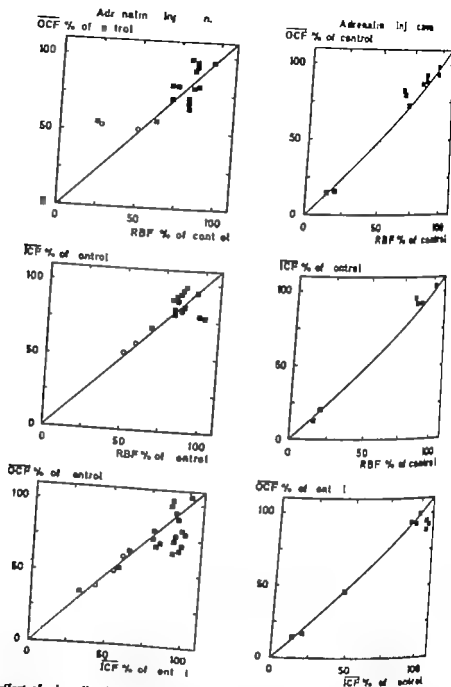


Fig. 3 The effect of adrenalectomy infused into the renal artery and the vena cava on the total renal blood flow (RBF) and the local cortical blood flow (OCF, ICF). Open circles. Renal arterial pressure lowered by aortic clamp.

**Intravenous infusion of vasopressor agents** The last control OCF and ICF prior to infusion of the different agents averaged  $3.37 (\pm \text{S.D. } 0.90)$  ml/min g and  $2.85 (\pm \text{S.D. } 0.87)$  ml/min g. Control RBF and  $\bar{A}P$  were  $3.70 (\pm \text{S.D. } 0.87)$  ml/min g and 109 mm Hg, respectively.

Intravenous infusion of the vasoactive agents caused similar flow changes as described above for intraarterial infusion. In Fig. 2 the mean flow of the 3 electrodes in the outer cortex

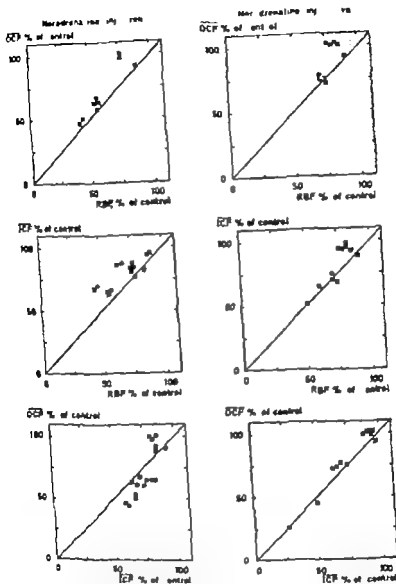


Fig. 4 The effect of noradrenaline infused into the renal artery and the vena cava on the total renal blood flow (RBF) and the local cortical blood flow (OCF/ICF). Open circles: Renal arterial pressure lowered by aortic clamp.

OCF) is plotted against the mean flow of 3 electrodes in the inner cortex (ICF) in the different experiments.

The OCF/ICF ratios in control measurements and during i.v. infusions of NA, Ang and ADH were 1.10 to 1.26, all significantly higher than unity. No significant change of the ratio was obtained during administration of NA, Ang and ADH (Table I). I.v. infusion of A caused a small, but statistically significant reduction in ratio, as did L.a. infusion.

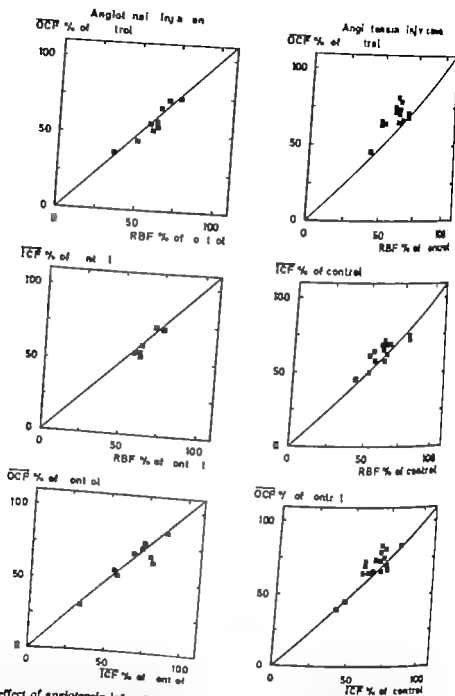


Fig. 5. The effect of angiotensin infused into the renal artery and the renal vein on the total renal blood flow (RBF) and the local cortical blood flow ( $\overline{OCF}$ ,  $\overline{ICF}$ ). Open circles. Renal arterial pressure lowered by aortic clamp.

The local control flow showed great variation between individuals. The results were therefore recalculated as percentage of the control flow to make values more comparable (Fig. 3-6). Typically flow at single electrode sites showed reductions essentially similar to the reduction of mean flow.

Whereas RBF could be completely stopped by high doses of NA, we were not able to

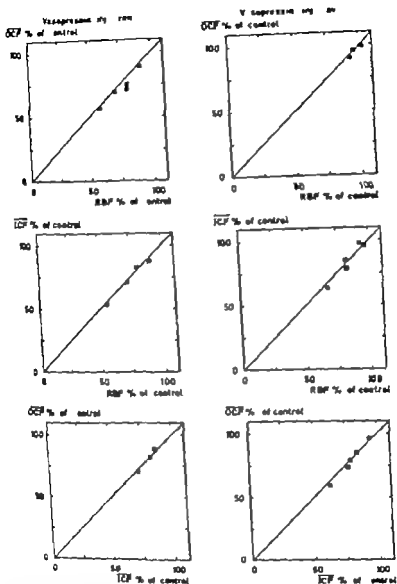


Fig. 6 The effect of vasopressin infused into the renal artery and the vena cava on the total renal blood flow (RBF) and the local cortical blood flow (OCF, ICF).

maintain a constant RBF of less than 25% of control. I.v. infusions of NA and Ang caused a marked rise in  $\bar{A}P$  but renal arterial pressure was held constant by adjustment of the aortic clamp.

The lowest RBF obtained by infusion of A, Ang and ADH were respectively 20%, 20% and 45% of control.

### Discussion

Flow per gram tissue measured with the microsphere method shows higher values in the outer cortex and lower in the inner cortex (Rector *et al* 1972) than those obtained in the present and earlier studies using the  $H_2$  clearance method (Aukland *et al* 1974, and Tysebotn 1974). This disagreement may be more apparent than real. The microsphere method measures glomerular flow and since flow per glomerulus is not very different in the various layers of the cortex (Horster, Kemler and Valtin 1971) more microspheres may be found in the outer cortex. On the other hand, washout of a diffusible substance such as  $H_2$  must be determined mainly by flow in the peritubular capillaries because of their much greater length and surface area compared to the glomerular capillaries. Since tubules in outer and inner cortex have much the same transport functions, there is a good reason to expect a great difference in peritubular blood flow in the two zones.

The main finding in the present study was a fairly proportional flow reduction in the outer and inner cortex with all the vasoconstrictors tested, indicating an evenly distributed constriction. The slightly greater fall in OCF than in ICF can hardly be of any physiological importance. Since cortical blood flow constitutes at least 70%, and probably closer to 80% of total renal blood flow, variations in average cortical flow should closely parallel changes in total renal blood flow (Barger and Herd 1973). This requirement is satisfied in the present findings (Fig. 3-6), thus providing indirect evidence for the validity of the  $H_2$  method for measuring total cortical flow.

While no measurements were made in the medulla in the present study, the proportional reduction of RBF and OCF and ICF with all 4 vasoconstrictors would suggest a proportional reduction also of medullary flow. However, because of the small flow fraction of the medulla, this reduction is liable to great errors, but agrees well with a proportional reduction of  $H_2$  clearance in outer medulla reported by Aukland (1968). Similarly, Gringsjo and Olsson (1971) found reduction of both cortical and medullary flow during infusion of Angiotensin II and high doses of NA, but the heat clearance method used did not permit any quantitative estimate of absolute or relative changes in blood flow. A qualitatively different result for which we have no explanation, was their finding of unaltered or even increased flow in medulla during i.v. infusion of small doses of NA.

While microsphere distribution differs from the present results during control conditions, as discussed above, Rector *et al* (1972) found equal relative reduction of microsphere uptake during infusion of NA and Ang in outer and inner cortex in good agreement with the present results.

As under several other experimental conditions completely different conclusions have been drawn from externally recorded washout of  $^{86}Kr$  or  $^{133}Xe$  and subsequent compartmental analysis. During infusion of NA (Carriere 1969, Grandchamp, Ayer and Trunfoglio 1971) the renal volume fraction corresponding to component I — assumed to represent outer cortex under control conditions, — is markedly reduced while the rate constant, indicative of flow per gram tissue is unchanged or slightly reduced. Component II normally considered to represent inner cortex and outer medulla usually shows increased flow rate and thus increased volume. Obviously the anatomical counterparts to these components change during vaso-



rections, and the interpretation given is that flow is reduced in outer cortex to such an extent that it becomes equal to an increased outer medullary flow thus accounting for the increase in the volume corresponding to component II. Flow in inner cortex is not much reduced and is represented by the now less voluminous component I. While this interpretation is supported by autoradiograms, no quantitative measurement of local gas concentration was prevented to validate the conclusions.

During washout of  $^{133}\text{Xe}$  Grandchamp *et al.* (1971) found a similar redistribution of blood flow during infusion of Ang (+Dibenzyliline) whereas Carriere and Friberg (1969) found a decrease in both cortical and medullary blood flow most pronounced in the subcapsular vascular region of the cortex. From the autoradiograms, they concluded that component I of the washout curve, believed to correspond to outer medulla under normal condition, represents regions of the cortex with a slower blood flow rate during Ang infusion. The rate of  $^{86}\text{Kr}$  was slower in outer medulla than in any part of the cortex.

The marked discrepancy between  $^{133}\text{Xe}$  or  $^{86}\text{Kr}$  washout and measurements of flow at comparable anatomical locations obtained with H<sub>2</sub> washout and other local recordings seems to be that external recording of  $^{133}\text{Xe}$  or  $^{86}\text{Kr}$  washout does not provide valid information on intrarenal blood flow distribution.

Of more interest is the autoradiographic demonstration of reduced uptake of  $^{86}\text{Kr}$  in the outer subcapsular zone during Ang infusion (Carriere and Friberg 1969). Since in the present study the electrodes in outer cortex were usually placed 1-3 mm underneath the skin surface, we cannot exclude a more pronounced vasoconstriction in most superficial cortical layer. On the other hand, we found no evidence for a patchy or segmental cortical vasoconstriction as indicated by white areas in  $^{86}\text{Kr}$ -autoradiograms (Carriere 1969; Grandchamp *et al.* 1971). The implications of the present results for renal excretory functions are not obvious. It has been theorized that reduction of salt excretion usually obtained during infusion of vasoconstrictor agents should be due to a redistribution of glomerular filtrate from superficial "salt losing" nephrons to deep "salt retaining" nephrons (Pomeranz, Lich and Berger 1968). However, no convincing experimental evidence for the existence of two such functionally different nephron populations has been presented, and the present study would rather suggest that salt retention should result from an even reduction of deep and superficial filtration rate and/or reduced peritubular capillary pressure.

The largest doses of vasoconstrictors used probably greatly exceed maximal endogenous secretion of these substances. However, combined effect of raised plasma concentrations of all these substances such as obtained, for instance during hemorrhagic hypotension (Cormick and Paladín 1964; Watts and Westfall 1964) might well be responsible for a slowing of renal vascular resistance. The finding of uniform distribution of blood flow during hemorrhagic hypotension (Aukland *et al.* 1973; Kjekshus and Tysebotn 1974) is therefore well consistent with the present results.

## References

- ANDERSSON, K. Effect of adrenaline, noradrenaline, angiotensin and renal nerve stimulation on intrarenal distribution of blood flow in dogs. *Acta physiol. scand.* 1968. 72, 498-509.
- ANDERSSON, K., A. KJELSHUS, E. LITWINSKY and I. TYSEBOTN. Effect of hemorrhagic hypotension on the distribution of renal cortical blood flow in anesthetized dogs. *Acta physiol. scand.* 1973. 87, 514-525.

- BARGER, A. C. and J. A. HERD, Renal vascular anatomy and distribution of blood flow in Ocel, Berliner and S. R. Geiger (Eds.), *Handbook of Physiology* Section 8: Renal Physiology. Am. Soc. Wash. 1973 269-313.
- CARRIERE, S., Effect of norepinephrine, isoproterenol, and adrenergic blockers upon the distribution of blood flow. *Canad. J. Physiol. Pharmacol.* 1969 47 199-208.
- CARRIERE, S. and J. FRIBORG, Intrarenal blood flow and PAH extraction during angiotensin. *Amer. J. Physiol.* 1969 217 1708-1715.
- GRANDCHAMP, A., G. AYER and B. TRUNGER, Pathogenesis of redistribution of intrarenal blood flow during hemorrhagic hypotension. *Europ. J. clin. Invest.* 1971 1 271-276.
- GRÄNGÅRD, G. and E. PERSSON, Influence of some vaso-active substances on regional blood flow in kidney. *Acta anaesth. scand.* 1971 15 71-95.
- HORSTER, M., B. J. KEMLER and H. VALTEN, Intracortical distribution of number and volume of glomeruli during postnatal maturation in the dog. *J. Clin. Invest.* 1971 50, 796-800.
- KIRKEBO, A. and I. TYKSEBOTN, Distribution of renal cortical blood flow during hemorrhagic hypotension in conscious dogs. *Acta physiol. scand.* 1974. 91 22-31.
- POMERANCE, B. H., A. G. BIRCH and A. C. BARGER, Neural control of intrarenal blood flow. *Physiol.* 1968. 215 1067-1081.
- RECTOR, J. B., J. H. STEIN, W. H. BAY, R. W. OSOONO and T. E. FOURIE, Effect of hemorrhagic hypotension on distribution of renal blood flow. *Amer. J. Physiol.* 1972. 222. 1125-1131.
- SCORNIACK, O. A. and A. C. PALADIN, Angiotensin blood levels in hemorrhagic hypotension related conditions. *Amer. J. Physiol.* 1964 206 553-556.
- TRUITT, R. J., A. E. BARCLAY, P. M. DANIEL, K. J. FRANKLIN and M. M. L. PERCHAUD, *Smaller arteries*. Blackwell Scientific Publ. Ltd. Oxford 1947.
- WATTS, O. T. and V. WESTFALL, Studies on peripheral blood catecholamine levels during hemorrhagic shock in dogs. *Proc. Soc. exp. Biol. (N.Y.)* 1964 115 601-604.

## Time Courses of Pulmonary Gas Exchange and Heart Rate Changes in Supine Exercise

By

HANS KARLSSON, BERTIL LINDBÖR and DAG LINNARSSON

Received 26 May 1975

### Abstract

KARLSSON, H., B. LINDBÖR and D. LINNARSSON. *Time courses of pulmonary gas exchange and heart rate changes in supine exercise*. Acta physiol. scand. 1975 95: 329-340.

In nine subjects of ventilation ( $\dot{V}_E$ ),  $O_2$  uptake ( $\dot{V}_{O_2}$ ),  $CO_2$  elimination ( $\dot{V}_{CO_2}$ ), respiratory exchange ratio ( $R$ ), end-tidal  $PO_2$  and  $PCO_2$  and heart rate (HR) were studied in seven subjects performing light dynamic exercises in the supine position. Individual and group mean time courses in response to step changes in work load were computed and displayed graphically. A computer-based method was also used to fit mono-exponential mathematical functions to the recorded responses. The over-all rate of HR change in response to the transition from 0-load pedalling to exercise (on-response) was faster (mean response time, MRT = 31 s) than the corresponding  $\dot{V}_{O_2}$  response (MRT = 45 s) while  $\dot{V}_E$  responded considerably slower (MRT = 86 s). During the reverse transition (off-response),  $\dot{V}_{O_2}$  and  $\dot{V}_E$  changed with the same rate as the on-response, while the HR-change was slower than during the on-response (MRT = 30 s). During a 15-sec period,  $\dot{V}_{O_2}$  changed only slightly - such contrasts to previous results in the sitting position, where 50% of the final change in  $\dot{V}_{O_2}$  has been reported to occur within the first 15-sec period, and how changes in blood distribution and stroke volume are known to be more pronounced than in the supine position. Our results emphasize the importance of central circulatory changes for the time course of  $\dot{V}_{O_2}$  at the start and end of exercise.

The time course of oxygen uptake ( $\dot{V}_{O_2}$ ) at the start of exercise can be considered to be the result of a multitude of adaptive changes of respiratory, circulatory and metabolic nature. Leitch, Gribert and Baule (1966) showed that variations in the response pattern of ventilation influenced the time course of gross  $\dot{V}_{O_2}$  but did not greatly alter the time course of the pulmonary-capillary  $O_2$  uptake, which was suggested to be mainly influenced by the dynamics of circulatory changes. In a recent study Linnarsson (1974) showed that two phases could be discerned in the time course of  $\dot{V}_{O_2}$  at the start of light exercise in the sitting position, a) a rapid initial change, assumed to result from an equally rapid readjustment of the blood flow through the lungs, b) a second rapid change after about 15 s, assumed to reflect the appearance of blood from the working muscles in the lungs. Each of the two phases constituted about 50 per cent of the final  $\dot{V}_{O_2}$  change. In the supine position, however, when changes in stroke volume, cardiac output and blood distribution are less marked (Bevegård, Hellgren and Jonsson 1960, Jones et al. 1970, Raynaud et al. 1973) the time courses of pulmonary gas exchange may differ from those at the onset of sitting exercise. It was therefore thought of interest to experimentally determine the time courses of pulmonary gas

TABLE I. Anthropometric data of subjects and work loads.

Subject	Age years	Weight kg	Height cm	Work load Watt
BL	24	64	176	107
TH	24	64	179	107
DL	29	61	172	98
BS	1	64	170	82
HK	27	61	176	82
MN	27	65	179	98
LF	32	81	189	107

exchange and heart rate changes in response to light to moderate exercise in the supine position

### Experimental and computational methods

**Subjects.** Seven healthy male sedentary subjects participated in the study. For individual data see Table I.

**Equipment.** The subjects exercised on an electrically braked cycle ergometer (Elema, Sweden) set for supine leg exercise (for details, see Wigeritz 1971). The work load could be automatically modified "zero load" (pedalling with no work load) to a preset work load in predetermined time intervals. Heart rate (HR) was obtained from chest electrodes and linear beat-to-beat cardiometer (Linn Wigeritz and Ödman 1969). The subjects breathed humidified room air through a respiratory valve with an expiratory outlet of the valve being connected to a Venturi-type flowmeter (Wigeritz 1969). A sample of the expired gas in the mouthpiece was drawn at a rate of 1 l/min through fast-responding  $O_2$  (Beckman model 209) and  $CO_2$  (Capnograph) analyzers. The outputs of the flow and gas concentration signals were fed into an analog computer (PACE TR-48), where pulmonary ventilation ( $\dot{V}_E$ ), oxygen ( $\dot{V}O_2$ ), carbon dioxide elimination ( $\dot{V}CO_2$ ) and respiratory exchange ratio (R) were computed beat-by-beat (Linnarsson and Lindberg 1974). These variables, together with HR and end-tidal concentrations of  $O_2$  and  $CO_2$  ( $P_{ET}O_2$  and  $P_{ET}CO_2$ , respectively) for each expiration obtained with an analog potentiometer (Carlsson *et al.* 1963), were displayed on an eight-channel analog ink-pen writer (Mark 200) and stored on a 14-channel FM tape recorder (AmpeX FR 100) for subsequent off-line processing of data. A flow chart showing the collection and the processing of data is given in Fig. 1.

**Experimental procedure.** The subjects performed supine exercise at a work load corresponding to approximately 45 per cent of maximal aerobic capacity (Table I) in 6-min periods alternating with 10-min zero-load pedalling (pedalling at 60 rpm with no work load imposed). Each experiment started with a 6-min warm-up period followed by four 10-min zero-load—6-min exercise periods, and successively thus could be made during four off-transients (two at zero-load transient) and four on-transients (load to work transient). In order to avoid influence of a deceleration on the measured responses, the load was changed without the subject's prior knowledge.

**Data processing.** The off-line data processing (Fig. 1) was performed by means of a digital computer. A computer with data acquisition capabilities (IBM 1800) was used for analog/digital conversion, correction and averaging of the recorded responses. The model estimation and data processing procedures were performed using a large computer (IBM 360/75).

Seven channels were transferred from the tape recorder to the computer: work load, HR,  $\dot{V}_E$  (corrected),  $\dot{V}CO_2$ ,  $P_{ET}O_2$  and  $P_{ET}CO_2$ . The sampling interval was 1 s. True R and true  $\dot{V}O_2$  are corrected for each sampling interval from  $\dot{V}CO_2$  and uncorrected  $\dot{V}O_2$  using the following relationships in corrected values in  $\dot{V}O_2$  due to  $\dot{V}_E/\dot{V}_E$  inequalities, when  $R \neq 1.0$ :

If

$$\text{measured } R = \frac{\dot{V}CO_2}{\text{measured } \dot{V}O_2}$$

then

$$\text{true } R = \frac{(1 - F_{I_{O_2}}) \text{ measured } R}{1 - F_{I_{O_2}}} \quad (\text{Linnarsson and Lindberg 1974})$$

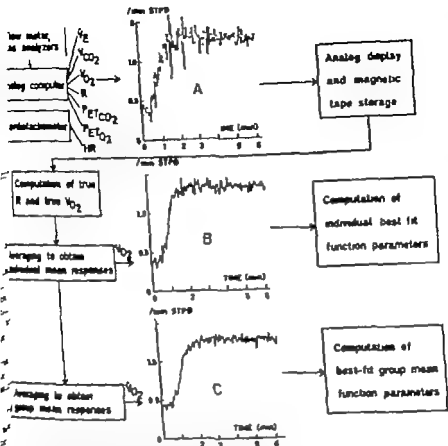


Fig. 1. Schematic diagram showing the analog and digital computer operations to compute, correct, display and parameterize the time course of  $\dot{V}_{O_2}$ . Curve A is a recording of single  $\dot{V}_{O_2}$  response of a subject. B is the average of 4 responses in the same subject, and curve C shows the group-mean response of 10 subjects.

$$\text{true } \dot{V}_{O_2} = \frac{\dot{V}_{O_2}}{\text{true } R}$$

For each subject and variable the 4 on- and the 4 off-responses were synchronized to the work load steps and averaged, so that each consecutive sample of the individual mean response was computed as average of 4 corresponding values of the recorded responses with identical time-relationships to the work load change. This procedure reduces the level (SD) of stochastic variations by a factor of 2, thus showing the basic time pattern of the response.

To remove short-lasting technical artefacts due to e.g. tape recorder drop-outs, an automatic artefact detection procedure was applied to the individual mean responses (Browna, Wigertz and Nygård 1970). A sample of the response was compared with the average of the values in a window symmetric around the sample. A specified number of the smallest and the largest values were removed. If the difference between the sample and the average was greater than a preset limit value the sample was replaced by the average. The window width, number of removed values, and difference limit value were individually set for each variable, dependent on response and disturbance characteristics.

To obtain the dynamic response characteristics for the entire group of seven subjects, group mean responses were computed using the same procedure as that used to obtain individual mean responses. The effect of this averaging is a reduction of the level (SD) of the stochastic variations by a factor of  $\sqrt{7}$  but also characteristic response patterns are smoothed if they occur time scattered in relation to the work load change.

To facilitate comparison between the responses of the different variables we attempted to find suitable parametric descriptions of the transients. Since some of the variables under study have been described to behave like exponential changes (i.e. Margaria, Edwards and Dill 1933, Cerretelli, Soland and Falcini 1963) mono- and bi-exponentially decaying functions were fitted to the responses. The functions used were of the form:

$$f(t) = \begin{cases} 0 & \text{for } t < T_D \\ a_1 [1 - \exp(-(t - T_D)/\tau_1)] & \text{for } t > T_D \end{cases} \quad \text{for } t < T_D$$

$$f_2(t) = \begin{cases} 0 & \text{for } t < T_D \\ a_1 [1 - \exp(-(t - T_D)/\tau_1)] + a_2 [1 - \exp(-(t - T_D)/\tau_2)] & \text{for } t > T_D \end{cases}$$

where  $a_1$  and  $a_2$  are amplitude coefficients,  $\tau_1$  and  $\tau_2$  (time constants) and  $T_D$  (time delay). Such a bi-exponential case is common to both time constants. The same fitting functions have been used earlier (Broman and Wigertz (1971) and Ljunger (1974)). The parameters were estimated by means of the least squares method, i.e. they were chosen to minimize the sum of squared differences between the function and the samples of the recorded response (loss function,  $L$ ). The variance of the differences can be estimated by  $S^2 = L/(N - n)$ , where  $N$  is the number of samples and  $n$  the number of estimated parameters. The fitting interval lasted from approximately 30 s before the change in work load until the next shift in work load. Since  $f(t)$  and  $f_2(t)$  are nonlinear with respect to time delay and time constants, an iterative procedure was required to minimize  $L$ . Several minimization procedures were tried and the method developed by Marquardt (1963) was found to be the most suitable.

The mean response time (MRT) can be used to indicate how fast a variable responds to a sudden change in the input function. For a monoexponential response, MRT is the sum of the time delay and the time constant. If the default value of a step response is defined as the area between the response and the steady state end value from the moment of work load change until steady state is reached, this also is equivalent to response amplitude multiplied by the mean response time (cf Whipp 1971, Ljunger 1974).

## Results

The group mean responses of  $V_D$ ,  $V_{O_2}$ ,  $V_O$ ,  $R$ ,  $P_{\text{resCO}_2}$ ,  $P_{\text{resO}_2}$  and HR to changes in work load from zero load to light exercise and in the reverse direction are depicted in Fig. 1 together with the best-fit functions of first or second order. Function parameters, i.e. best values of amplitudes, time constants and time delays for the group-mean responses are presented in Table II together with mean values of the function parameters from individual responses.

*Configuration of the individual and group-mean curves.* Some typical response patterns consistently occurred in the individual responses of HR and  $V_{O_2}$ . The on-response of HR always displayed an initial steep increase with no delay followed after 10–20 s by a levelled off or a transient decrease of 5–10 s duration. Thereafter HR increased at a rate considerably slower than the initial response. No such "notch" in the HR response was observed in the off transient. A fast initial increase of small amplitude, sometimes with an overshoot, followed by a levelling-off period and a secondary rise, was observed also for  $V_{O_2}$  in all subjects. After the cessation of work  $V_{O_2}$  remained increased for about 15 s, before a rapid fall took place. These typical individual response patterns occurred sufficiently uniform in relation to the work load changes to appear also in the group-mean responses (Fig. 2, 4).

*Steady state values.* Group-mean values for the last 30 s period of each condition have been determined for all variables and these values have been included in the computation of the MRT values for the on- and off responses.

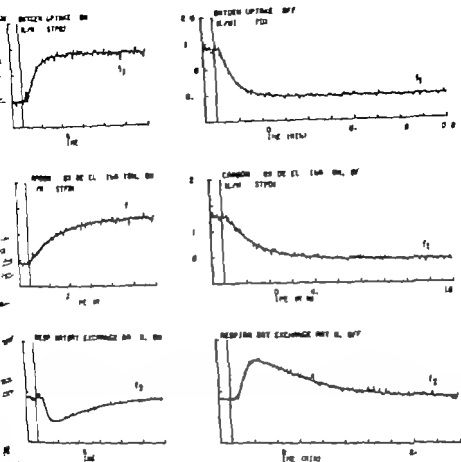


FIG. 2. Graphical representation of group means of oxygen uptake, carbon dioxide elimination and respiratory exchange ratio to light exercise. Responses from both the transition between load-pedalling and exercise (on) and the reverse transition (off) are shown. Each tracing computed from beat-by-beat recordings from 11 subjects in 7 subjects. 0-time denotes the time for the change in work rate. Best-fit functions of first ( $f_1$ ) or second order ( $f_2$ ) are superimposed on the recorded responses.

$\dot{V}_{O_2}$  during zero-load pedalling amounted to 0.49 l/min STPD and the corresponding  $R$  was 92.2 beats/min.  $\dot{V}$  averaged 13.0 l/min BTPS which, as judged from the  $R$  and  $\dot{V}_{CO_2}$  values (0.95 and 36 mm Hg, respectively), may have been a slight hyperventilation. At the end of the exercise period  $\dot{V}_{O_2}$  averaged 1.41 l/min yielding an average work efficiency of 0.30, which is in agreement with the findings of Whipp and Wassermann (1969), who also examined work efficiency during light exercise with zero load as the baseline condition, but used conventional Douglas-bag technique for the  $\dot{V}_{O_2}$  determinations. HR during the last 30 s of exercise averaged 123.3 beats/min, whereas  $\dot{V}$  amounted to 31 l/min BTPS. End-tidal  $P_{aO_2}$  was 5 mm Hg lower and end-tidal  $P_{aCO_2}$  was 6 mm Hg higher than during zero-load pedalling.

First-order exponential functions could be fitted to both the on- and the off-responses of

To obtain the dynamic response characteristics for the entire group of seven subjects, group means were computed using the same procedure as that used to obtain individual mean responses. The effect of this averaging is a reduction of the level (SD) of the stochastic variations by a factor of  $1/\sqrt{7}$  but the characteristic response patterns are smoothed if they occur time scattered in relation to the work load change.

To facilitate comparison between the responses of the different variables we attempted to find parametric descriptions of the transients. Since some of the variables under study have been shown to behave like exponential changes (*i.e.* Margaria, Edwards and Dali 1933, Corricelli, Sakad and Farkas 1960) mono- and bi-exponentially decaying functions were fitted to the responses. The functions used were of the form:

$$f_1(t) = \begin{cases} 0 & \text{for } t < T_D \\ a_1[1 - \exp(-(t - T_D)/\tau)] & \text{for } t \geq T_D \end{cases} \quad \text{for } t < T_D$$

$$f_2(t) = \begin{cases} 0 & \text{for } t < T_D \\ a_1[1 - \exp(-(t - T_D)/\tau)] + a_2[1 - \exp(-(t - T_D)/\tau_2)] & \text{for } t \geq T_D \end{cases}$$

where  $a$  and  $a_1$  are amplitude coefficients,  $\tau$  and  $\tau_2$  time constants and  $T_D$  is time delay. Such an exponential case is common to both time constants. The same fitting functions have been used by Broman and Wigertz (1971) and Linnarsson (1974). The parameters were estimated by means of a least squares method, *i.e.* they were chosen to minimize the sum of squared differences between the fitted samples of the recorded response (loss function,  $L$ ). The variance of the differences can be estimated by  $S^2 = L/(N - n)$ , where  $N$  is the number of samples and  $n$  the number of estimated parameters. The fitting interval lasted from approximately 30 s before the change in work load until the next shift in load. Since  $f_1(t)$  and  $f_2(t)$  are nonlinear with respect to time delay and time constants, an iterative procedure was required to minimize  $L$ . Several minimization procedures were tried and the method developed by Marquardt (1963) was found to be the most suitable.

The mean response time (MRT) can be used to indicate how fast a variable responds to a sudden change in the input function. For a monoexponential response, MRT is the sum of the time delay and time constant. If the deficit value of a step response is defined as the area between the response and the steady state value from the moment of work load change until steady state is reached, then deficit is response amplitude multiplied by the mean response time (*cf.* Whipple 1971, Linnarsson 1974).

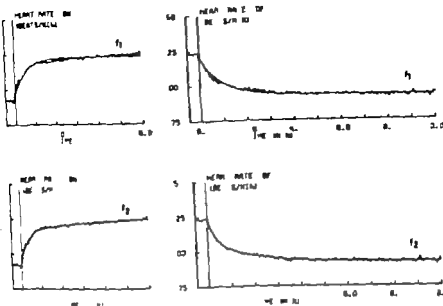
## Results

The group mean responses of  $V_E$ ,  $V_{O_{2max}}$ ,  $\dot{V}_{O_2}$ ,  $\dot{V}_{O_2}/V_{O_{2max}}$ ,  $\dot{V}_{O_2}/V_{O_{2max}}$ ,  $\dot{V}_{O_2}/V_{O_{2max}}$ , and HR to changes in work load from zero load to light exercise and in the reverse direction are depicted in Figure 1 together with the best-fit functions of first or second order. Function parameters, *i.e.* values of amplitudes, time constants and time delays for the group-mean responses are listed in Table II together with mean values of the function parameters from individual responses.

*Configuration of the individual and group-mean curves.* Some typical response patterns consistently occurred in the individual responses of HR and  $\dot{V}_{O_2}$ . The on-response always displayed an initial steep increase with no delay followed after 10–20 s by a levelling off or a transient decrease of 5–10 s duration. Thereafter HR increased at a rate considerably slower than the initial response. No such "notch" in the HR response was observed off-transient. A fast initial increase of small amplitude, sometimes with an overshoot followed by a levelling-off period and a secondary rise, was observed also for  $\dot{V}_{O_2}$  in all subjects. After the cessation of work  $\dot{V}_{O_2}$  remained increased for about 15 s, before a rapid fall took place. These typical individual response patterns occurred sufficiently uniform in the group to the work load changes to appear also in the group-mean responses (Fig. 2, 4).

*Steady-state values.* Group-mean values for the last 30 s period of each condition have been determined for all variables and these values have been included in the computation of the deficit values for the on- and off-responses.





4. Group mean on- and off-responses of heart rate together with first- (upper tracing) and second-order (lower tracing) best-fit functions. The superior fitting of the second-order function is clearly shown.  $\delta$  values and conditions as in Fig. 2.

as the fitted first-order functions. Thus both the on- and off-responses of HR were better described by second-order functions, as also is shown by the much lower  $\delta$  values in this set. This relationship is visualized in Fig. 4, where both first and second-order best-fit functions are graphically represented together with the measured responses.

**Time delays.** The time courses, described in terms of time constants and time delays, showed marked differences between the various variables. The general tendency was that variables with the slowest on-response also exhibited the slowest off-response, but except for  $\dot{V}_{O_2}$  no symmetry between on- and off-responses was observed. Negative time delays were found in the best-fit first-order functions for  $\dot{V}$  and HR, suggesting that the order of applied functions were too low since, when a second-order function was applied to HR,  $T_D$  approached zero (Table II). Positive time delays averaging between 12.5 and 22.8 s were found in the on- and off-responses of  $\dot{V}_{O_2}$ ,  $R$ ,  $P_{KRCO_2}$  and  $P_{KRCO_2}$  and in the off-responses of  $\dot{V}_E$  and  $\dot{V}_A$ .

**Time constants.** The first-order time constants ranged from 28.8 s ( $\dot{V}_{O_2}$  on) to 91.8 s ( $\dot{V}_E$  on). The second-order functions describing the biphasic responses of  $R$  and  $P_{KRCO_2}$  contained one short time constant ( $\tau_1$ ) and one 5–9 times longer time constant ( $\tau_2$ ) having opposite direction. For the second-order description of the mean HR responses,  $\tau_1$  was approximately the same for the on- and off-transients, while  $\tau_2$  differed markedly and was 4.5 times longer in the off-transient. This slow change, with  $\tau_2 = 455$  s and with an asymptote of 130.8 beats/min, may also be described as a linear change of HR averaging  $+1.2$  beats  $\text{min}^{-1}$  during the last 10 min of exercise.

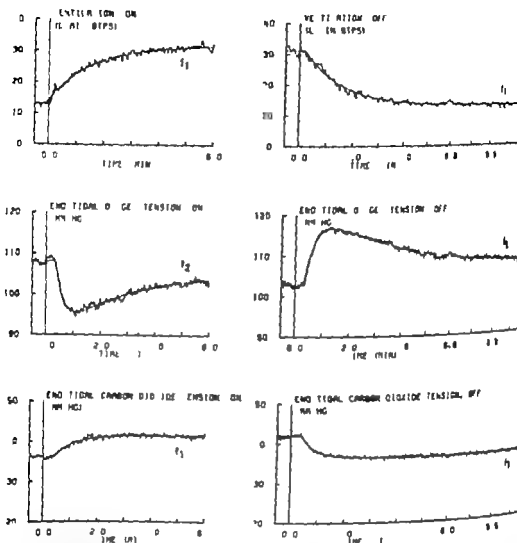


Fig. 3 Group mean on- and off-responses of ventilation, end-tidal  $P_{O_2}$  and end-tidal  $P_{CO_2}$ . Symbols conditions as in Fig. 2.

$V_E$ ,  $\dot{V}_{O_2}$ ,  $\dot{V}_{O_2}$ , and  $P_{ETCO_2}$ , both on an individual and on a group basis. Attempts to fit second-order functions to these 4 variables were made but no second time constants could be estimated. The values of the function parameters of the group-mean responses closely approximated the mean values of individual parameters in the majority of cases. Group averaging reduced the  $S$  value approximately in proportion to the number of subjects, indicating that the reduction of  $S$  obtained with the averaging procedure was accomplished mainly through the reduction of stochastic variations.

Second-order functions were required to describe the biphasic on- and off-response patterns of  $R$  and  $P_{ETCO_2}$ . The fitting procedure failed with individual data in one or more of the subjects, so only the function parameters of the group-mean responses are given in Table 1.

Both first and second-order functions could be fitted to the group-mean on- and off-responses of HR. Group averaging did not reduce the first-order  $S$  values in proportion to the number of subjects, indicating that the experimental HR responses systematically differed

on- and off-responses (MRT approx 45 s) did not differ significantly nor did oxygen debt (0.67 l STPD) differ significantly from oxygen debt (0.74 l STPD) (cf. above, "Data missing").

### Discussion

The main purpose of the present investigation was to study the interrelationships between the time courses of the  $\dot{V}_{O_2}$  uptake and the circulatory changes occurring during the unsteady state of exercise. Oxygen uptake measured breath-by-breath at the mouth was used to assess the dynamics of the uptake of  $\dot{V}_{O_2}$  by the circulation in the lungs, and beat-by-beat heart-rate readings were made to assess the corresponding adjustments of cardiac output. In situations when large changes in pulmonary  $\dot{V}_{O_2}$  stores take place, resulting from changes in functional residual capacity (FRC) and/or in alveolar  $P_{O_2}$ , there will be a transient difference between  $\dot{V}_{O_2}$  and the pulmonary-capillary  $\dot{V}_{O_2}$  uptake (Ancliminos, Gilbert and Baule 1966). During the first 30 s of light work in the sitting position, FRC decreases by about 1 l (Lindansson 1974) but no data seem to be available concerning FRC changes at the start and end of work in the supine position. The expiratory reserve volume (ERV) during passive rest is, however, substantially decreased compared to erect position (Sjodstrand 1951, Jones *et al.* 1962) and it seems reasonable to assume, therefore, that only slight, if any, FRC changes take place at start and end of light supine work. Alveolar  $P_{O_2}$  is virtually unchanged during the initial 15–20 s period following the work-load change as reflected by end-tidal values in the present study (Fig. 3, Table II) and by arterial measurements (Jorres and Wigertz 1971). It can therefore be assumed that  $\dot{V}_{O_2}$  reflects with reasonable accuracy the pulmonary-capillary  $\dot{V}_{O_2}$  uptake during the initial 15–20 s of light supine exercise recovery while during the following 15–20 s period, when large changes in  $P_{ET,O_2}$  take place,  $\dot{V}_{O_2}$  will differ from pulmonary-capillary  $\dot{V}_{O_2}$  uptake. From the observed  $P_{ET,O_2}$  changes this difference can be calculated to be some 0.1 l/min STPD representing a transient underestimation of pulmonary-capillary  $\dot{V}_{O_2}$  uptake at the start of work and a corresponding overestimation at the end of work.

It is well established that in the supine position the stroke volume during steady state of isometric exercise does not differ greatly from resting values (Bevegard, Holmgren and Nelson 1960, Jones *et al.* 1970). Raynaud *et al.* (1973) showed that this was true also for the steady state of exercise with the exception for the initial 30 s period after the onset of work, when the stroke volume exhibited a transient decrease by some 10 per cent. An opposite change of similar small magnitude has been observed during the first minutes of recovery from light supine exercise, when stroke volume has been reported to be increased by 10–20 per cent (Cunningham 1972). Heart-rate changes can therefore be considered as fair estimates of the adjustments of cardiac output under the conditions of the present study.

### Over-all rates of response

Broman and Wigertz (1971), studying relatively well-trained subjects, found MRT values of about 15 s for the on-response of HR under conditions almost identical to that of the present study. The value of about 30 s for the sedentary subjects of the present study resembles the findings of Broman and Wigertz when using a much higher absolute work load, and this may indicate that the over-all rate of readjustment of HR is functionally coupled to the

TABLE II Parameterization of best-fit first and second-order functions for ventilation ( $\dot{V}_E$ , l/min BTPS), carbon dioxide elimination ( $\dot{V}_{CO_2}$ , l/min STPD), oxygen uptake ( $\dot{V}_{O_2}$ , l/min STPD), respiratory exchange ratio (R), end-tidal  $P_{CO_2}$  ( $P_{ETCO_2}$ , mm Hg), end-tidal  $P_{O_2}$  ( $P_{ETO_2}$ , mm Hg) and heart rate (HR, beats/min). Data refer to transients following onset of work (on) and recovery (off). Time constants ( $\tau$ , s) and time delay ( $T_D$ ) are given in sec. Group mean responses (mean  $\pm$  SE) as well as mean values  $\pm$  standard error of the mean of the individual responses (see values) are shown for each variable and parameter. Indicates that second-order model could be fitted to the group mean responses but not to all of the individual responses.

	$\mu$	$\tau$	$\mu_2$	$\tau$	$T_D$	$S^2-W^2$
<b>on</b>						
$\dot{V}_E$	18.8 19.3 $\pm$ 1.5	91.8 104.5 $\pm$ 26.1			-5.8 -4.7 $\pm$ 5.2	48 347
$\dot{V}_{CO_2}$	0.87 0.88 $\pm$ 0.06	84.2 86.0 $\pm$ 11.2			2.7 3.7 $\pm$ 4.9	877 529
$\dot{V}_{O_2}$	0.90 0.91 $\pm$ 0.03	28.6 29.3 $\pm$ 3.6			16.1 14.6 $\pm$ 3.8	154 766
R	-0.35	14.7	0.37	145.9	22.2	883
$P_{ETCO_2}$	5.6 5.7 $\pm$ 0.7	45.9 43.6 $\pm$ 5.1			23.2 22.9 $\pm$ 4.8	101 643
$P_{ETO_2}$	-17.2	16.4	14.3	151.6	21.8	326
HR	30.3 30.5 $\pm$ 2.8	35.7 35.8 $\pm$ 4.9			-4.9 -4.6 $\pm$ 1.1	1654 3185
HR	4.8	20.3	13.8	455	-1.7	436
<b>off</b>						
$\dot{V}_E$	-17.8 -17.8 $\pm$ 1.5	81.7 80.2 $\pm$ 5.2			12.5 14.6 $\pm$ 3.8	389 2280
$\dot{V}_{CO_2}$	-0.84 -0.84 $\pm$ 0.05	71.9 71.0 $\pm$ 4.5			12.5 13.6 $\pm$ 2.5	862 352
$\dot{V}_{O_2}$	-0.94 -0.93 $\pm$ 0.03	33.5 32.3 $\pm$ 1.5			14.1 15.3 $\pm$ 1.7	862 371
R	0.70	29.0	-0.69	133.1	21.0	6182
$P_{ETCO_2}$	-5.6 -5.7 $\pm$ 0.7	30.4 40.3 $\pm$ 16.6			22.8 21.4 $\pm$ 3.2	883 616
$P_{ETO_2}$	20.3	29.4	-15.9	172	20.0	351
HR	-30.3 -30.5 $\pm$ 2.7	56.6 56.4 $\pm$ 6.3			-6.2 -6.7 $\pm$ 1.7	1852 3832
HR	-15.4	17.2	-15.7	101.3	0.8	428

The mean response time (see above "Data processing") represents the time for the "set of gravity" of the transient under study and can be used to compare the rates of adjustment of the various variables.  $\dot{V}_E$  and  $\dot{V}_{CO_2}$  were the slowest changing variables both in the on- and off-transients with MRT values between 85 and 95 s. The on-transients for HR represent the fastest change (MRT = appr. 30 s) while its off-transient was 20 s slower ( $p < 0.01$ ). F

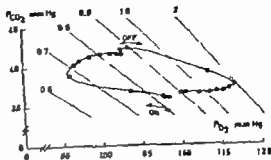


Fig. 1.  $O_2$ - $CO_2$  diagram showing the changes in end-tidal  $P_{O_2}$  versus end-tidal  $P_{CO_2}$  during the first 5 min of exercise (on) and recovery (off). Filled circles denote start values and open circles indicate every 30 s. The uppermost R-slopes show that end-tidal  $R$  was generally more than 0.1  $R$  units above the corresponding  $R$  slope calculated from  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$  (cf. Fig. 2).

rapid changes versus time delays in the initial responses of HR and  $\dot{V}_{O_2}$ .

An immediate rapid HR increase at the onset of exercise was originally described by Krogh and Lindhard (1913) and it was thought to be associated with a rapid rise in the  $O_2$  uptake. In the present study HR responded immediately and 50 per cent of the HR change had on the average taken place during the first 10–15 s period. The concomitant small increase of  $\dot{V}_{O_2}$  (1 l/min) was of a magnitude that would be found if the oxygen pulse (i.e. the product of stroke volume and a-v  $O_2$  difference) had not changed during the same period. This is compatible with the findings of Raynaud *et al.* (1973), who observed only a slight increase in the a-v  $O_2$  difference 10 s after the onset of supine exercise paired with a corresponding slight transient decrease in stroke volume. In the present study the major part of the response of  $\dot{V}_{O_2}$  was observed after a delay of about 15 s. After that time  $\dot{V}_{O_2}$  increased more rapidly than HR and cardiac output, indicating a widening of the arterio-venous  $O_2$  difference due to a drop in the mixed venous  $O_2$  content (Raynaud *et al.* 1973). Provided that the onset of work is associated with an immediate fall in  $P_{O_2}$  in the blood perfusing the working muscles, the above delay will reflect the time necessary for the blood to traverse the vascular segment between working muscles and the lungs.

The off-transient of  $\dot{V}_{O_2}$  showed no change at all until about 15 s after the work-load stopped. Deoxygenated blood, returning during the first half circulation time after the end of exercise, may prevent a rapid fall of  $\dot{V}_{O_2}$  and if at the same time, venous return is sustained, no fall at all of  $\dot{V}_{O_2}$  would be observed as in the present case. The post-exercise increase in stroke volume, reported by Cumming (1972) to take place in supine subjects, may have contributed to maintain venous return despite the observed rapid fall in HR at the end of work, and so may probably the loadless pedalling movements by activating the muscle pump in the legs.

## References

- BOUDREAU, J. H. J. R. GILBERT and G. H. BAKER, Effect of ventilation on oxygen transfer during early exercise. *J. appl. Physiol.* 1966 21 810–818.
- BRUNNEN, S. A. HOLMGRAN and B. JONSSON, The effect of body position on the circulation at rest and during exercise, with special reference to the influence on the stroke volume. *Acta physiol. scand.* 1960, 24, 279–298.
- KEITH, H. and B. WIGGERS, Dynamics of arterial oxygen tension in response to muscular work load in man. *Acta physiol. scand.* 1971 82, 236–249.
- JOHANSSON, I. and O. WIGGERS, Transient dynamics of ventilation and heart rate with step changes in work load from different load levels. *Acta physiol. scand.* 1971 81, 54–74.

relative rather than the absolute work load. A comparison of the off-responses of  $\dot{V}_E$  between the two studies yields similar conclusions. The observation that off-responses of  $\dot{V}_E$  were slower than the corresponding on-responses is also in accordance with previous observations (Cerretelli, Sikand and Farhi 1966, Gilbert, Auchincloss and Baile 1967, Broman and Wigertz 1971). The over-all ventilatory responses were similar to those reported by Broman and Wigertz, and it was confirmed that the mean response of  $\dot{V}_E$  is considerably faster than that of  $\dot{V}_O_2$  both in the on- and the off-transients.

Our finding that the increase in  $\dot{V}_E$  leads that of  $\dot{V}_O_2$  is in accordance with the findings of Cerretelli *et al.* (1966). This does not exclude, however, that the peripheral oxygen uptake readjusts at the same rate as, or even slightly faster than cardiac output, as was found by Gilbert, Auchincloss and Baile (1967).

The mean response time (45 s) for the on response of  $\dot{V}_O_2$  in the present study corresponds to a half time of 30 s, which is the value commonly found among previous investigators at the transition from rest to exercise (Henry and de Moor 1956, Margaria *et al.* 1965, Cerretelli *et al.* 1966, di Prampero *et al.* 1970, Whipp 1971, Whipp and Wasserman 1972).

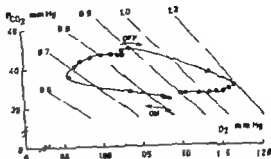
The congruence between the on- and off-responses of  $\dot{V}_O_2$  is by definition associated with an O deficit/O debt ratio close to unity. This is in agreement with Whipp, Seard and Wasserman (1970) who concluded that, using zero load as the base-line condition before and after steady-state submaximal work, the efficiency for anaerobic and aerobic work is equal. The present results thus support the concept that the fast component of the  $\dot{V}_O_2$  rise consists of repayment of loans from stores of molecular O and phosphagens (adenosine triphosphate and creatinephosphate), that were utilized at the onset of exercise.

There was a striking similarity between the time course of  $\dot{V}_E$  and  $\dot{V}_O_2$ , both with regard to short term and long term changes. The similarity in the short term fluctuations suggests passive behavior of  $\dot{V}_O_2$ , in a sense that rapid changes of CO stores are induced by  $\dot{V}_E$  fluctuations. The similarity in the long term changes suggests a tight functional coupling between the production of  $\text{CO}_2$  and the response of  $\dot{V}_E$  to submaximal work (cf. Wasserman, Van Kessel and Burton 1967).

### *Responses of end-tidal $P_{O_2}$ , $P_{CO_2}$ and R*

The discrepancies between the time courses of  $\dot{V}_{CO_2}$  and  $\dot{V}_O_2$  result in large changes of R following the onset and the termination of exercise (Fig. 2). These changes of R can be visualized together with the corresponding changes in the end-tidal gas tensions in an  $\text{O}_2$ - $\text{CO}_2$  diagram (Fig. 5) (Rahn and Fenn 1955). Using the terminology of these authors, the changes of end-tidal  $P_{O_2}$  versus  $P_{CO_2}$  can be described as a hypoventilation loop constituting the dominant response during exercise and recovery. The end-tidal R values of the diagram appear generally to be about 0.1 lower than those obtained from gas exchange measurements (Fig. 2), which discrepancy may reflect differences between end-tidal and mean alveolar  $P_{O_2}$  due to the cyclic  $P_{O_2}$  and  $P_{CO_2}$  changes within each breath (Mateff 1963, Rosenhamer 1972).  $P_{O_2}$  falls and  $P_{CO_2}$  rises towards the end of an expiration with an associated decrease of the instantaneous R value (Kim, Rahn and Farhi 1966) and may thus explain the observed differences between mean alveolar R and end tidal R.

$O_2$ - $CO_2$  diagram showing the changes of  $P_{CO_2}$ , venous end-tidal  $P_{O_2}$  during a 5 min ill exercise (oe) and recovery. Filled circles denote start values and circles indicate every 30 s. The upper and lower isopleths show that end-tidal  $R$  generally were some 0.1  $R$  units than the corresponding  $R$  values computed from  $\dot{V}_{CO_2}$  and  $\dot{V}_{O_2}$  (cf. Fig. 7).



at changes: cross time delays in the initial responses of HR and  $\dot{V}_{O_2}$ .

Immediate rapid HR increase at the onset of exercise was originally described by Krogh and Lindhard (1913) and it was thought to be associated with a rapid rise in the  $O_2$  uptake. In the present study HR responded immediately and 50 per cent of the HR change had on the average taken place during the first 10–15 s period. The concomitant small increase of  $\dot{V}_{O_2}$  initially was of a magnitude that would be found if the oxygen pulse (i.e. the product of stroke volume and a-v  $O_2$  difference) had not changed during the same period. This is compatible with the findings of Raynaud *et al.* (1973), who observed only a slight increase in a-v  $O_2$  difference 10 s after the onset of supine exercise paired with a corresponding transient decrease in stroke volume. In the present study the major part of the response of  $\dot{V}_{O_2}$  was observed after a delay of about 15 s. After that time  $\dot{V}_{O_2}$  increased more slowly than HR and cardiac output, indicating a widening of the arteriovenous  $O_2$  difference and to a drop in the mixed venous  $O_2$  content (Raynaud *et al.* 1973). Provided that the onset of work is associated with an immediate fall in  $P_{O_2}$  in the blood perfusing the working sites, the above delay will reflect the time necessary for the blood to traverse the vascular circuit between working muscles and the lungs.

The off-transient of  $\dot{V}_{O_2}$  showed no change at all until about 15 s after the work-load stopped. Deoxygenated blood, returning during the first half circulation time after the end of exercise, may prevent a rapid fall of  $\dot{V}_{O_2}$  and  $\dot{M}$  at the same time, venous return is maintained, no fall at all of  $\dot{V}_{O_2}$  would be observed as in the present case. The post-exercise increase in stroke volume, reported by Cumming (1972) to take place in supine subjects, may have contributed to maintain venous return despite the observed rapid fall in HR at the end of work, and so may probably the loadless pedalling movements by activating the muscle pump in the legs.

### References

- LOVINGOOD, J. H. J., R. GILBERT and O. H. BAULE, Effect of ventilation on oxygen transfer during bicycling. *J. appl. Physiol.* 1968 21 810–818.
- LEVILAIN, S., A. HOLMÖR and B. JOHANSSON, The effect of body position on the circulation at rest and during exercise, with special reference to the influence on the stroke volume. *Acta physiol. scand.* 1960, 81, 279–290.
- LEVILAIN, H. and O. WIKSTRÖM, Dynamics of arterial oxygen tension in response to isometric work load. *Acta physiol. scand.* 1971 82 234–249.
- JACOBSEN, S. VILMANN and O. WIKSTRÖM, Transient dynamics of ventilation and heart rate with step changes in work load from different load levels. *Acta physiol. scand.* 1971, 87 54–74.

- BROMAN, S., ■ WIGERTZ and M. E. NYGÅRD, A procedure for reduction of noise and artefacts in automatic processing of physiological measurement data. *Rep. Lab. A-lar Naval Med., Karol. Inst., Stockholm* 1970. 32 pp.
- CARLSSON, A., G. HANNAUER, T. CAREY and P. J. HOLMES, *Handbook of Analog Computation*. Electronic Associates, Inc., Princeton, New Jersey 1965. 389 pp.
- CERRITELLI, P., R. SIKAND and L. E. FARHI, Readjustments in cardiac output and gas exchange during onset of exercise and recovery. *J. appl. Physiol.* 1966. 21. 1345-1350.
- CUMMING, G. R., Stroke volume during recovery from supine bicycle exercise. *J. appl. Physiol.* 1970. 28. 575-578.
- GILBERT, R., J. H. AUCHINCLOSS, Jr and G. H. BAULE, Metabolic and circulatory adjustments to metabolic state exercise. *J. appl. Physiol.* 1967. 22. 905-912.
- HANDON, J. S., B. S. TABAKIN and E. J. CALDWELL, Response of lung volumes and ventilation to postural change and upright exercise. *J. appl. Physiol.* 1962. 17. 783-786.
- HENRY, F. M. and J. C. DeMOORE, Lactic and alactic oxygen consumption in moderate exercise of postural intensity. *J. appl. Physiol.* 1956. 8. 608-614.
- JONES, W. B., R. N. FOMCHUM, R. O. RUSSELL, Jr and T. J. REEVE, Transient cardiac output response to multiple levels of supine exercise. *J. appl. Physiol.* 1970. 28. 183-189.
- KROGH, A. and J. LINDBHARD, The regulation of respiration and circulation during the initial steps of muscular work. *J. Physiol. (Lond.)* 1913. 51. 112-136.
- KIM, T. S., H. RAHN and L. E. FARHI, Estimation of true venous and arterial  $P_{O_2}$  by gas analysis of single breath. *J. appl. Physiol.* 1966. 21. 1338-1344.
- LINDBORG, B., O. WIGERTZ and TH. ÖDMAN, A beat-to-beat heart-rate meter with linear endoglycophorin for muscular exercise studies. *Rep. Lab. A-lar Naval Med., Karol. Inst., Stockholm* Dec. 1968. 3 pp.
- LINNARSSON, D., Dynamics of pulmonary gas exchange and heart rate changes at start and end of recovery. *Acta physiol. scand.* 1974. Suppl. 413.
- LINNARSSON, D. and B. LINDBORG, Breath-by-breath measurement of respiratory gas exchange in on-line analog computation. *Scand. J. clin. Lab. Invest.* 1974. 34. 219-224.
- MARGARIA, R., H. T. EDWARDS and D. B. DILL, The possible mechanisms of contracting and post-contracting oxygen debt and the role of lactic acid in muscular contraction. *Amer. J. Physiol.* 1933. 906. 689-703.
- MARGARIA, R., F. MANGILI, F. CUTTICA and ■ CERRITELLI, The kinetics of the oxygen consumption at onset of muscular exercise in man. *Ergonomics* 1965. 8. 49-54.
- MARQUARDT, D. W., An algorithm for least squares estimation of nonlinear parameters. *J. Soc. Ind. Appl. Math.* 1963. 11. 431-441.
- MATTELL, G., Time-courses of changes in ventilation and arterial gas tensions in man induced by moderate exercise. *Acta physiol. scand.* 1963. 28. Suppl. 206.
- DI PRAMPERO, P. E., C. T. M. DAVIES, P. CERRITELLI and R. MARGARIA, An analysis of  $O_2$  debt component in submaximal exercise. *J. appl. Physiol.* 1970. 29. 547-551.
- RAHN, H. and W. O. FIDIC, *A Graphical Analysis of the Respiratory Gas Exchange*. Washington, D.C. Amer. Physiol. Soc. 1933. 38 pp.
- RAYNAUD, J., H. BERNAL, J. P. BOURDARIAS, P. DAVID and J. DURAND, Oxygen delivery and oxygen consumption in the lungs at onset of exercise in man. *J. appl. Physiol.* 1973. 35. 59-62.
- ROSENHAMER, ■, Estimation of hyaloid  $P_{O_2}$ . *Acta physiol. scand.* 1972. 84. 22. 2.
- SÖSTRAND, T., Determination of changes in the intrathoracic blood volume in man. *Acta physiol. scand.* 1951. 22. 114-128.
- WASSERMAN, K., A. L. VAN KESSEL and G. G. BURTON, Interaction of physiological mechanisms during exercise. *J. appl. Physiol.* 1967. 22. 71-83.
- WHIPP, B. J., Rate constant for the kinetics of oxygen uptake during light exercise. *J. appl. Physiol.* 1970. 30. 261-263.
- WHIPP, B. J., C. SEARD and K. WASSERMAN, Oxygen deficit-oxygen debt relationships and efficiency in anaerobic work. *J. appl. Physiol.* 1970. 28. 452-456.
- WHIPP, B. J. and K. WASSERMAN, Efficiency of muscular work. *J. appl. Physiol.* 1969. 26. 644-648.
- WHIPP, B. J. and K. WASSERMAN, Oxygen uptake kinetics for various intensities of constant-load work. *J. appl. Physiol.* 1972. 33. 351-356.
- WIGERTZ, ■, A low-resistance flow meter for wide-range ventilatory measurement. *Respir. Physiol.* 1971. 7. 63-70.
- WIGERTZ, O., Dynamics of respiratory and circulatory adaptation to muscular exercise in man. *Acta physiol. scand.* 1971. 82. Suppl. 363.



## Influence of Substance P on the Response of Guinea Pig Ileum to Transmural Nerve Stimulation

By

■ HEDQVIST and U. S. VON EULER

Substance P (SP) seems to be closely associated with nervous tissue of various kinds. Thus, presence of SP in intestine has been correlated with the presence of intrinsic nerve plexuses in intestinal wall (Pernow 1953) and large amounts of SP have been found in the dorsal root ganglia of sensory nerves (Lembeck 1953), and in other nerves (Pernow 1953, Euler 1963). SP depolarizes motoneurons (Otsuka, Konishi and Takahashi 1972), sensory nerves (Lembeck 1973) and certain neurons within the central nervous system (Krnjević and Morris 1974, Phyllis and Linacher 1974), it has been attributed a transmitter function and a role as modulator of nerve activity.

Locally oligodynamic concentrations of SP were found to increase the tone and to enhance the stimulus response in the guinea pig vas deferens (Euler and Hedqvist 1974). In the present study we wish to report on the influence of synthetic SP on responses of the circular muscle layer of the guinea pig ileum to transmural nerve stimulation and to acetylcholine (ACh).

Guinea pigs were killed by a blow on the head and the longitudinal muscle layer of the ileum was carefully dissected free and placed in a 5 ml bath with Tyrode solution (for composition, see Hedqvist and Euler 1972) aerated with 5% CO<sub>2</sub> in O<sub>2</sub>. Field stimulation was applied through platinum electrodes in the wall of the bath. The preparation, loaded with 0.25 g, was stimulated every minute with 1-2 pulses at 2 Hz through a Grass S4 Stimulator in order to elicit a twitch-like contraction. The pulse duration was 1 ms and the voltage suprathreshold. Contractions were recorded isotonically using a Harvard heart/smooth muscle transducer coupled to a Honeywell 190 inkwriter. Synthetic SP was kindly placed at our disposal by Professor S. Leeman and used in aqueous solution of 1 or 10 µg/ml.

Single pulses were in most cases sufficient to elicit a brisk contraction. The stimulation parameters, as well as the fact that the contraction response was abolished by atropine ( $10^{-6}$  M) and left unchanged by guanethidine ( $3 \cdot 10^{-6}$  M), phentolamine ( $6 \cdot 10^{-6}$  M) and propranolol ( $7 \cdot 10^{-6}$  M) indicate activation of cholinergic nerves.

A concentration of  $1.5 \cdot 10^{-10}$  M SP regularly enhanced the contraction response to transmural nerve stimulation and often increased the basal tone of the preparation. Increasing the SP dose up to  $7.5 \cdot 10^{-10}$  M caused a dose-dependent increase in basal tone whereas the enhancement of induced contractions remained the same or increased mod-

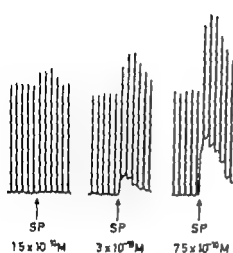


Fig. 1

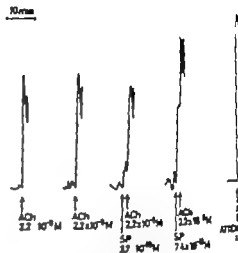


Fig. 2

Fig. 1. Strip of longitudinal muscle layer from guinea pig ileum. Effect of SP on contraction resp. transmural nerve stimulation (2 pulses, 2 Hz, 1 ms, supramaximal voltage) resumed each sec.

Fig. 2. Strip of longitudinal muscle layer from guinea pig ileum. Effect of SP on contraction resp. a standard dose of ACh,  $2.2 \cdot 10^{-6}$  M. To the right maximal contraction response (attained 1 ACh).

erately (Fig. 1). Both effects were of relatively short duration inasmuch as they reached maximum within 2–3 min and usually vanished after an additional 5 min. On the other hand, SP in the same concentrations as those found to enhance responses to transmural stimulation only occasionally and weakly enhanced contractions induced by ACh. In most cases the contraction response to ACh remained unchanged or even decreased in the presence of SP (Fig. 2).

Since the contractile effect of SP on intestinal smooth muscle is not affected by atropine or hexamethonium, an action directly on effector cells has been assumed (Persson 1960). Similarly, Lewis (1960) found the stimulant effect of SP on the gut to be much less than that of agents which act by stimulating nervous structures. On the other hand, Beleslin and Varagic (1960) found that SP sometimes enhanced the contractions of the guinea pig ileum induced by ACh. Since hexamethonium inhibited this enhancement, they concluded the effect of SP to be partly due to sensitization of ganglionic cells.

The present finding that SP much more readily enhanced contractions of the guinea pig ileum resulting from nerve stimulation than from ACh administration suggests a prejunctional action of SP on cholinergic transmission, in addition to the direct stimulant action on the smooth muscle cells. The high potency of SP together with its presence in the gut wall invites the speculation of SP as a physiological modulator of smooth muscle in this tissue.

This study was supported by grants from the Swedish Medical Research Council, project no. 64 and from Stiftelsen Lars Hiertas Minne.

### References

- BELESLIN, D. and V. VARAGIC, The effect of Substance P on the responses of the isolated guinea-pig to acetylcholine, nicotine, histamine and 5-hydroxytryptamine. *Arch. int. pharmacodyn.* 1960. 1, 327.

12. U. E. Substance P in subcellular particles in peripheral nerves. *Ann. N.Y. Acad. Sci.* 1963. 84, 448-461.
13. U. E. and P. HEDQVIST. Effects of Substance P on the response of guinea pig vas deferens to chemical stimulation. *Acta physiol. scand.* 1974. 90, 451-452.
14. JENSEN, P. and U. E. EULER. Prostaglandin induced neurotransmission failure in the field-stimulated, relaxed vas deferens. *Neuropharmacology* 1972. 11, 177-187.
15. J. H. and F. LIDBACK. Effect of substance on perivascular pain receptors of the perfused rabbit ear 'Vamp-Schmiedberg' *Arch. Pharmacol.* 1973. 277 R. 34. Suppl.
16. JENSEN, K. and M. E. MORRIS. An excitatory action of Substance P on cutaneous neurons. *Canad. J. Physiol. Pharmacol.* 1974. 52, 736-744.
17. LIDBACK, F. Zur Frage der zentralen Übertragung afferenter Impulse. III. Mitteilung. Das Vorkommen und die Bedeutung der Substanz P in den dorsalen Wurzeln des Rückenmarks. *Vamp-Schmiedberg Arch. exp. Path. Pharmacol.* 1953. 219, 197-213.
18. G. P. The inhibition by morphine of the action of smooth muscle stimulants on the guinea-pig intestine. *Br. J. Pharmacol.* 1960. 15, 425-431.
19. KIKU, M., S. KONOJO and T. TAKAHASHI. A further study of the motoneuron-depolarizing peptide extracted from dorsal roots of bovine spinal nerves. *Proc. Jap. Acad.* 1972. 48, 747-752.
20. KROW, R. Studies on Substance P: Purification, occurrence and biological actions. *Acta physiol. scand.* 1951. 29, Suppl. 105.
21. ALLEN, J. W. and J. J. LIDBACK. Substance P excitation of cerebral cortical Beta cells. *Brain Res.* 1974. 88, 158-161.

## Lactate Concentration in Type I and II Muscle Fibres during Muscular Contraction in Man

By

BIRGITTA ESSÉN and TOM HÅGMARK

Human skeletal muscles have two main fibre types, type I and type II. Both have glycogen content, with glycolytic activity being more pronounced in type II fibre. The potential in type I may be enough to maintain a high rate of glycolysis. A recruitment pattern of the different fibre types may exist at onset of work and varies of exercise (Grimby and Hannerz 1968, Gollnick, Piehl and Saltin 1974).

It has not been established whether an accumulation of lactate occurs in both fibres found in human skeletal muscle or mainly in the type II fibres. To investigate this a method to determine lactate in fragments of single muscle fibres or fibres of the same type is introduced. In this report the method will be described together with preliminary results from experimental procedures known to result in a lactate production.

5 healthy subjects participated in the study. 3 subjects performed maximal bicycle exercise. Biopsies were taken from each subject at 2 different situations in separate experiments. As indicated in Fig. 1, biopsies were obtained at rest and after 10, 20 and 40 s after start of exercise and at exhaustion (3-4 min). One subject performed work of maximal intensity with a pressure cuff placed round one leg (300 mmHg). Biopsies were obtained from both legs at exhaustion (80 s). Two subjects performed isometric work at 30% of MVC. Biopsies were taken in separate experiments after 20 s and at exhaustion (1 min). 1 and 5 is the same person.

The muscle biopsies were taken from vastus lateralis (Bergström 1962) and frozen in liquid nitrogen within 2-4 s.

An enzymatic fluorimetric method was used for analyzing lactate. The reagent solution consisted of 100 mM glycine buffer, 100 mM hydrazine-hydroxide, 1 mM NAD and lactic dehydrogenase. The reaction was started by adding an aliquot of the sample. Concomitant blanks and standards were used. The reaction time was 15-20 min.

Twenty-five biopsy samples containing lactate (1.5-4.5 mmol/kg wet weight) were divided into two portions. One portion was extracted in 3 M HClO<sub>4</sub> as in earlier studies while the other portion was freeze-dried and extracted in 1 M HCl.

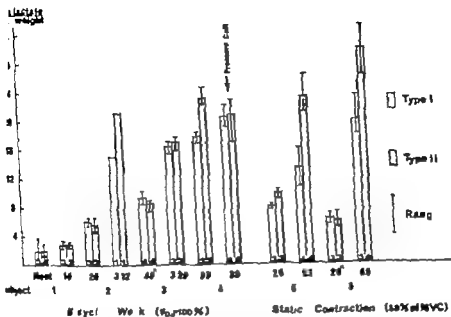
No significant difference was seen in lactate concentration between the two extraction methods ( $p < 0.05$ ). When HClO<sub>4</sub>-extraction was performed at 100°C for 2 h no change in lactate concentration was seen ( $p < 0.05$ ). This would enable the determination of both lactate and glycogen from the same sample (Essén and Henriksson 1974).

### Analysis of lactate in pooled fibres

Muscle samples were freeze-dried and single fibres dissected out and identified as type I or II by ATPase staining as described earlier (Essén *et al.* 1975). 25-50 fibres of the same type were pooled, weighed (25-75 µg) and placed in 15 µl of 1 M HCl for 20 min. 8 µl of the supernatant was then added to 2 ml reagent solution and analyzed for lactate as above.

### Analysis of lactate in individual fibres

150 µl of reagent solution were used in this assay. After the first reading on the fluorometer 1 µl of the sample was added to the inner wall of the tube above the reagent solution and a single fibre (1-3 µg) was added.



1. Lactate concentration is shown in type I and type II fibres in human skeletal muscle during rest and at different times during bicycle work at maximal level and static contractions at 50% of maximal voluntary contraction (MVC). 6 subjects participated and 2 different samples were taken from each subject. Number within each bar indicates the number of samples of pooled fibres that were analysed. Notes: values are expressed as at eight ascending water content of 71% in the muscle.

the drop of HCl. The reaction was then started by adding the drop of HCl to the reagent solution after 25 min. 25 fibres were divided and lactate determined on each half. The coefficient of variation of the values was 18%.

The lactate concentration at rest and work on pooled fibre types with the method used in this study agrees well with results from the literature (Saltin *et al.* 1971; Diamond, Karlsson & Saltin 1968).

At rest the lactate concentration was 1.8 mmol/kg wet weight in both fibre types (Fig. 1). After 10 s of intense dynamic work the concentration had increased to 2.8 mmol/kg wet weight in both fibre types. A further rise in lactate concentration occurred during the work and reached 20 mmol/kg wet weight at exhaustion (3–4 min).

One of the subjects had a higher concentration in the type II fibres (only one pool from each fibre type). In the leg with blood flow occluded there was equal high lactate concentrations in both fibre types during maximal work (20 mmol/kg wet weight). The non-occluded leg had a 5 mmol/kg wet weight higher lactate concentration in type II fibres (the range for the samples does not overlap). The high lactate concentration in the leg with intact flow may be due to asymmetric division of work output between the two legs.

After 10 s of isometric contraction lactate concentration in both fibre types was similar but at exhaustion type II fibres had 10 mmol/kg wet weight higher lactate concentration than type I fibres in both subjects (the range for the samples does not overlap). The fact that type II fibres have a higher concentration of lactate may be related to their function.

SUBJECT NO.	EXPERIMENT	TYPE I		TYPE II	
		SINGLE FIBRES	POOLED FIBRES	SINGLE FIBRES	POOLED FIBRES
4	Dynamical work	15.8 ± 2.1	17.6	25.9 ± 6.5	22.9
	Exhaustion time	(13.5-18.4)	(16.7-18.2)	(16.2-34.8)	(22.1-34.8)
		$n_1$ 5	2 3	$n_1$ 11	$n_2$ 3
4	Dynamical work	25.4 ± 5.1	20.2	26.7 ± 4.5	16.6
	Exhaustion time with pressure on/off	(15.0-32.9)	(19.0-21.9)	(21.0-35.1)	(17.8-22.8)
		$n_1$ 10	$n_2$ 6	$n_1$ 21	$n_2$ 6
5	Static contraction (50 MVC) 20	8.3 ± 3.8	7.9	12.1 ± 2.8	9.7
		(6.0-15.5)	(7.6-8.2)	(6.2-15.7)	(5.8-14.8)
		$n_1$ 16	2 3	$n_1$ 20	$n_2$ 3
5	Static contraction (50 MVC) 33	18.8 ± 4.9	13.2	27.2 ± 3.6	23.3
	Exhaustion	(7.2-23.6)	(10.6-18.8)	(19.5-30.5)	(20.9-24.8)
		$n_1$ 13	2 4	$n_1$ 11	$n_2$ 4
6	Static contraction (50 MVC) 70	9 ± 2.5	6.2	7.0 ± 2.8	5.8
		(3.1-13.8)	(5.4-6.9)	(3.6-11.5)	(4.9-7.5)
		$n_1$ 18	2 3	$n_1$ 16	$n_2$ 3

TABLE I. Mean value, SD and range of lactate concentration in single fibres ( $n_1$  number of fibres, range in parentheses) and mean value for pooled fibres ( $n_2$  number of samples of pooled fibres). Dynamical work: 100%  $\dot{V}O_2$  and static contraction: 50% of maximal voluntary contraction.

Lactate was also determined on individual fibres in five biopsies and the mean value well with those found on pooled samples, however a very marked variation (possibly analytical error) was observed within each fibre type (Table I).

As pointed out in the introduction a selective recruitment of muscle fibres (fibre type) may occur in exercise. The large variation found for the lactate concentration of the individual muscle fibres agrees well with only certain muscle fibres being activated. A possibility that at least part of the lactate found in some fibres may have diffused from an adjacent lactate producing muscle fibre.

This study was supported by grant from the Swedish Medical Research Council (04X-4554).

## References

- BERGSTROM, J. Muscle electrolytes in man. *Scand. J. Clin. Lab. Invest.* 1962. Suppl. 68.  
 DIAMANT, B., J. KARLSSON and B. SALTIN, Muscle tissue lactate after maximal exercise in man. *Acta physiol. scand.* 1968. 72. 383-384.  
 ERIÉN, B. and J. HENRIKSSON, Glycogen content of individual muscle fibres in man. *Acta physiol. scand.* 1974. 90. 645-647.  
 ERIÉN, B., E. JANSSON, J. HENRIKSSON, A. W. TAYLOR and B. SALTIN, Metabolic characteristics of types in human skeletal muscle. *Acta physiol. scand.* 1975. 95. 153-165.  
 GOLLNICK, D., A. FIEHL and B. SALTIN, Selective glycogen depletion pattern in human muscle after exercise of varying intensity and at varying pedalling rates. *J. Physiol. (Lond.)* 1974. 241. 1-11.  
 GRIMBY, L. and J. HANSSON, Recruitment order of motor units on voluntary contractions, changes by proprioceptive afferent activity. *J. Neurol. Neurosurg. Psychiatr.* 1968. 31. 565-573.  
 SALTIN, B., P. D. GOLLNICK, B. O. ERIKSSON and K. FRIML, Metabolic and circulatory adjustments of maximal work. In: *Order of Exercise*, eds. A. Gibert and P. Guille, Univ. of Toulouse, 1971.

# Effect of Stretch on Potassium Contracture Tension in Twitch and Slow Muscle Fibres of *Xenopus laevis*

By

JAN LÄNNERGREN

Depolarization of the surface membrane of a skeletal muscle fibre initiates a series of events which ultimately leads to contraction of the fibre. The relation between membrane potential and force generation has been investigated by Hodgkin and Horowitz (1960) in isolated frog twitch fibres by rapidly changing the external potassium concentration,  $[K]_o$ , and recording tension production associated with the ensuing step in membrane potential. The log  $[K]_o$ -tension relation is described by a steep S-shaped curve, starting at about  $-50$  mV (20 mM K) and reaching a maximum at about  $-20$  mV (80 mM-K). It has recently been reported (Lázaro-Serrano, Valle and Cifero 1973) that extending a frog twitch muscle fibre from 2 to  $\pm 2.8$   $\mu$ m sarcomere length increases the tension response to depolarizations by fractional K-concentrations (30-60 mM) by about 25%. The present report shows that a similar effect is obtained in *Xenopus* twitch fibres but not in slow muscle fibres.

Single fibres were dissected from the skeletal muscle of medium-sized clawed toads (*Xenopus laevis*) under artificial illumination. The absence of light scattering particles was used as a guide for selecting fibres of different fractional types (Lännergren and Smith 1964). For the twitch fibre experiments, granular fibres located outside the toad's bundle were selected. For the slow fibre experiments, transparent fibres near the bases of the toad's bundle were used. Fibres selected to be of the twitch type were tested for their ability to give twitches and tetanus on electrical stimulation. The response of slow fibres was tested at the beginning of each experiment, the criterion for correct identification being local contraction on electrical stimulation and large, sustained contracture in 20 mM-K solution.

The selected fibre was mounted in a narrow chamber with one tendon held fixed and the other connected to a piezoelectric force transducer (AE 801, Akers Electronics, Horten, Norway). The resonance frequency of the transducer (in air) was 2 kHz and its compliance  $0.013$  m  $N^{-1}$ . The fibre could be observed through a high-power microscope lens suspended in the chamber. A cover-slip was placed on top and a Leitz UMK 30 objective was used. Measurements of sarcomere length were made with the aid of a calibrated ocular scale. Various solutions are introduced into the chamber by means of a stop-cock system. Ringer solution (NaCl 115 mM, KCl 2.5 mM,  $CaCl_2$  2.0 mM, phosphate buffer 3.0 mM, pH 7.2) was continuously flowing at a slow rate through the chamber between tests. A contracture of the fibre was induced by a quick change (1-2 s) from Ringer solution to a solution of increased K-concentration. Contracture solutions were prepared in such a way as to keep the  $[K]_o$   $[Cl]_o$  product constant (Hodgkin and Horowitz 1959). The experiments were performed at room temperature (21-24°C).

Fig. 1A and B show the effect of stretch from 2.25  $\mu$ m (A) to 3.05  $\mu$ m (B) sarcomere length on the mechanical threshold of a *twitch* fibre. The K-concentration used was 17.5 mM which was sub-threshold at the shorter length but elicited a relatively large contracture (25% of

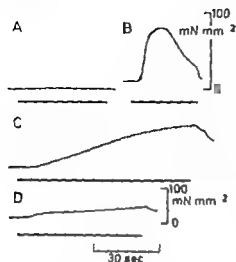


Fig. 1 Tension developed by a twitch fibre at 2.25  $\mu$ m sarcomere length (A), and at 3.05  $\mu$ m (B) in 17.5 mM-K solution applied during time indicated by horizontal bar. C, time course of tension of a slow fibre in 12.5 mM-K solution at 3.15  $\mu$ m sarcomere length, D in same solution at 3.15  $\mu$ m

maximum tetanic tension) at the greater length. With larger positive polarizations tensions developed also at the shorter length but up to about 30 mM K larger responses were obtained after the fibre was stretched. Above 30 mM K the tension response was reduced by stretching the fibre. This was expected since it is well-known that the maximum tension is related to the amount of filament overlap (Gordon, Huxley and Julian) and overlap is decreased by stretching the fibre beyond 2.2  $\mu$ m sarcomere length. The relation between  $[K]_o$  and peak contracture tension for twitch fibres at two sarcomere lengths is presented in Fig. 2A. The results obtained on *Xenopus* twitch fibres agree with the data obtained by Gonzales-Serratos *et al.* on frog twitch fibres. The displacement to the left of the  $[K]_o$ -tension curve after stretch was larger than that observed by them, but so was the displacement to the right (35% of rest length as compared to 15–20% in their case). This suggests that even larger effects might be obtained by greater extensions.

Records C and D of Fig. 1 show the effect of stretch on the mechanical performance of a slow fibre at just suprathreshold positive polarization. In contrast to the twitch fibre the slow fibre response was larger at the shorter length. This was true also for all other positive polarizations (Fig. 2B). The effect of stretch on the mechanical threshold was somewhat difficult to determine since tension development was very slow at low K-concentrations but it was quite clear that a threshold-lowering effect like that seen in twitch fibres was absent in slow fibres.

Possible mechanisms behind the stretch effect in twitch fibres have been discussed by Endo (1972) who suggested that the Ca-sensitivity of the myofilaments is increased by stretch and by Gonzales-Serratos *et al.* (1973) who ascribed the effect to increased release of Ca from intracellular stores.

The present investigation reveals a clear-cut difference in response to stretch of fast and slow *Xenopus* muscle fibres. Slow fibres differ from twitch fibres in several respects: (1) the mechanical threshold is lower and the  $[K]_o$ -tension relation steeper (Lännergren 1972), (2) the dependence of tension on  $[Ca]_i$  is stronger (Lüttgau 1963), and (3) the sensitivity towards replacement of extracellular Ca by other divalent ions is greater (Edwards and Vaughan 1967). These differences most likely reflect differences in excitation-Ca release coupling.



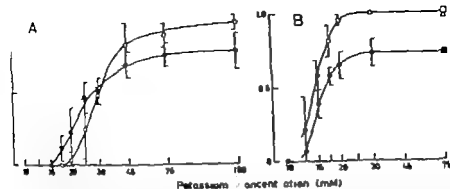


Fig. 2. Relation between peak contracture tension and  $[K_+]$  at 2 different sarcomere lengths, 2.25  $\mu\text{m}$  (open circles) and 3.05  $\mu\text{m}$  (filled circles). The symbols represent mean values from measurements on four fast fibres (O) and four slow fibres (●) (except at 75 mM-K where single measurements are given). Vertical bars indicate  $\pm$  S.D. Ordinate also 1.0 is tetanic tension (75–100 Hz) at 2.25  $\mu\text{m}$  sarcomere length and 70 mM-K contracture tension (2.25  $\mu\text{m}$ ) in B.

tempting to assume that the difference in the response to stretch is also due to a difference in the activation mechanism but it cannot be excluded that the properties of the contractile states of the two fibre types are different.

Investigation was supported by the Swedish Medical Research Council (Project No. 14X 3642) and grants from Karolinska Institute's Fonder.

### References

- WANG, C. and H. LOUCCOVIC, The role of calcium in excitation-contraction coupling in various species of the frog, mouse, and barnacle. *Amer. Zoologist* 1967 7 615–622.
- WANG, M., Stretch-induced increase in activation of skinned muscle fibres by calcium. *Nature New Biol.* 1972 237 211–213.
- WILLIAMS-SCHAFER, H., R. VALLS and A. CALDERO, Effect of muscle stretching on tetanic development and mechanical threshold during contractions. *Nature New Biol.* 1973 246 221–222.
- WILSON, A. M., A. F. HINDLEY and P. J. JULIAN, The variation in isometric tension with sarcomere length in vertebrate muscle fibres. *J. Physiol. (Lond)* 1966 124 170–192.
- WOLFE, A. L. and P. HOROWITZ, The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol. (Lond)* 1959 148 127–140.
- WOLFE, A. L. and P. HOROWITZ, Potassium contractures in single muscle fibres. *J. Physiol. (Lond)* 1960 131 346–403.
- WOLFE, A. L., Contractures of single slow muscle fibres of *Xenopus laevis* elicited by potassium, acetylcholine or choline. *Acta physiol. scand.* 1967 89 362–372.
- WOLFE, A. L. and R. S. MATH, Types of muscle fibres in toad skeletal muscle. *Acta physiol. scand.* 1966 89 203–214.
- WOLFE, A. L., The effect of calcium on potassium contractures of single muscle fibres. *J. Physiol. (Lond)* 1961 144 679–687.

## Behavioural Signs of Emotional Excitement from Fastigial Striatum in "Chronically" Decerebrate Cats

By

BJÖRN LILANDER and JAN MARTNER

Stimulation of the cerebellar fastigial nucleus can elicit sham-rage in thalamic cats, an effect which is abolished by acute precollicular decerebration (Zanchetti and Zoccolato 1971). These findings can be taken as evidence of excitatory connections from the cerebellar fastigial nucleus to the hypothalamic neuron pools involved in aggressive behaviour. It is, however, known that also mesencephalic or even pontile cats can display signs of rage or fear, albeit not as integrated as the behaviour patterns seen in hypothalamic animals (Bard and Mach 1936). Several days have to elapse after decerebration before these responses can be observed. As is the case in hypothalamic cats, they can be elicited by sound or nociceptive stimuli, suggesting that influences causing sham-rage exert at least part of their action at low stem levels, possibly *via* fairly unspecific actions on reticular neurons. It was therefore considered of interest to study whether fastigial stimulation is capable of eliciting emotionally coloured behavioural patterns also in chronically decerebrate cats.

Six cats were operated under combined ketamine and pentobarbital anesthesia, under strict aseptic precautions. The right carotid artery and the right external jugular vein were cannulated with silicone rubber catheters with their tips placed in the aortic arch and the right atrium, respectively. The other catheter ends were drawn out through the skin on the back of the skull. The latter was opened over the occipital lobes which were then removed, permitting egress of cerebrospinal fluid through the lateral ventricles. The spinal cord was then cut at the predetermined level by a blunt spatula and the wound was closed. In subsequent days the animals received electrolytes, glucose solution, amino acid solution and lipid suspension i.v. The arterial catheter was flushed daily with heparin solution. Heart rate and serum potassium and sodium were followed and if necessary corrected. Overheating was prevented by a fan, regulated by a rectal thermistor keeping body temperature at about 35°C, which was increased to 37-38°C during the observations of behaviour. After 3-5 days the animals were lightly anesthetized with pentobarbital and the fastigial nucleus (for references see Martner 1975) was stereotactically localized and stimulated during recording of blood pressure. The electrode was cemented in place. After at least 24 hours the electrode was again stimulated and the behaviour of the animals was observed. All animals treated in this way were kept for 6-7 days after decerebration and their brains were fixed in formaldehyde for subsequent macroscopic and histological examination.

of the cats the brain stem was transected through the lower mesencephalon and in the cats at the junction between the mesencephalon and the pons. Strong extensor rigidity appeared within a few hours after decerebration and tonic neck and labyrinthine reflexes readily elicited. When undisturbed, the animals lay on their side. Some days after the operation they displayed righting of the head, often induced by handling. Some of the animals could right their (ore)limbs as well as their head.

Four of the animals, emotionally coloured response patterns were observed and in two of these occurred "spontaneously" or was easily triggered by touch or vibration of the body. In the two other animals such behaviours were elicited first when nociceptive stimuli were applied. The responses consisted of attempts to right head and shoulders, protrusion of the tongue, running movements with all legs, lashing of the tail and an increased respiratory rate. In one cat there was vocalization as well. Increases in heart rate and blood pressure and contraction of the nictitating membrane paralleled these somatomotor changes.

Three of the animals were implanted with an electrode in the fastigial pressor area. Stimuli (square waves, 50 Hz, 1 ms and 0.03–0.2 mA) caused marked increases in blood pressure, heart rate and all the animals displayed the above described somatomotor changes, in part or partly. Thus, all animals made running movements with their legs and increased respiratory rate and three of them righted themselves with head and shoulders and readily tried to escape. One low mesencephalic animal reacted very vividly had to be prevented from falling off the table, growled and urinated. These reactions were entirely stimulus-bound and as a rule ceased within 20 s after stimulation. As a rebound phenomenon, yawning and chewing movements were regularly observed.

Stimulations causing marked pressor responses induced a somatomotor pattern, entirely of the above described type and postural reactions (Hare, Magoun and Ranson 1937) were apparent, neither during nor after stimulation. If stimulation intensity was decreased, minimal responses could be observed initially in some animals, only to be followed by the emotionally coloured changes. In deteriorated preparations, in which the emotional responses no longer could be evoked by nociceptive stimuli, postural changes were seen both during and, as a rebound phenomenon, after the stimulus, especially at intense stimulation. In two additional animals were decerebrated acutely under ether anaesthesia. Fastigial pressor stimulation caused, as expected, increased blood pressure and heart rate. However the emotionally coloured somatomotor changes observed in the chronically decerebrate cats did not be induced, except for an increased respiratory rate.

In agreement with earlier findings, the present experiments have shown that even decerebrate animals can display fragments of flight or attack behaviour though these responses are not as well integrated and easily elicited as in hypothalamic animals (Bard and Macht 1946). The somatomotor changes were paralleled by autonomic changes such as increases in heart rate and blood pressure and retraction of the nictitating membranes.

Similar behavioural changes could be elicited by stimulation of the fastigial pressor area. These findings suggest that although fastigial stimulation can induce aggressive behaviour in thalamic animals, this cannot be taken as evidence of direct cerebellar projections to the diencephalon. The fact that acute decerebration can prevent these behavioural responses may be explained by the inflicted trauma (Monakow 1914, p. 26). It is unclear

whether the state in "chronically" decerebrate animals represents a recovery to normality of brain stem structures or whether supersensitivity develops. Whichever the present study has demonstrated cerebellar projections to bulbar and mesencephalic neuron pools involved in emotional behaviour possibly the same which can be seen in intact animals or hypothalamic preparations.

This research has been sponsored by grants from the Swedish Medical Research Council (N) and by grants from the Medical Faculty of Göteborg.

### References

- BARD, P. and M. B. MACHY: The behaviour of chronically decerebrate cats. In: *Cerebral Foundations of the Neurological Basis of Behaviour*. Ed. by H. W. Wolstenholme and C. M. O'Connor. J. & A. Churchill, London, 1958, 55-75.
- HALL, W. K., H. W. MARSH and S. W. RANSOM: Localization within the cerebellum of reaction to cerebellar stimulation. *J. comp. Neurol.* 1937, 67, 145-162.
- MARTNER, J.: Cerebellar influences on autonomic mechanisms. An experimental study in the rat with special reference to the fastigial nucleus. *Acta physiol. scand.* 1975, Suppl. 425.
- MONAKOW, C. von: *Die Lokalisation im Grosshirn und der Aufbau der Funktionen durch Lese*. J. F. Bergmann, Wiesbaden, 1914, 1, 1033.
- ZANCHETTI, A. and A. ZOCOLINI: Autonomic hypothalamic outbursts elicited by cerebellar stimulation. *J. Neurophysiol.* 1954, 17, 475-483.

## Reversibility of Effects of Very Hypotonic Fluids on *In Vitro* Frog Gastric Mucosa. A Functional and Morphological Study

By

H. F. HELANDER, S. S. SANDERS, L. L. SHANBOUR and W. S. REHM

Received 19 December 1974

### Abstract

HELANDER, H. F., S. S. SANDERS, L. L. SHANBOUR and W. S. REHM. *Reversibility of effects of very hypotonic fluids on in vitro frog gastric mucosa. A functional and morphological study* Acta physiol. scand. 1975. 95. 353-363.

Functional and morphological properties of the *in vitro* frog gastric mucosa were studied during and after exposure to very hypotonic ( $< 25$  mOsm) solutions. Within 20 min the acid secretory rate decreased to zero, but it returned to normal levels after mucosal fluids had been restored. The potential differences (PDs) dropped within the first minutes after the exposure to hypotonic solutions, and became inverted. Following the return of isotonic conditions the PD increased to levels higher than in the controls. The electrical resistances increased about 10-fold during the hypotonic period, but decreased to near normal values when isotonic conditions were restored. By light and electron microscopy the cells of the hypotonic mucosa appeared greatly swollen, and the alterations were assessed by morphometric methods. The gland lumens were almost obliterated, and the luminal protein was reduced to about 60% of its former volume. After the return to isotonic conditions normal morphology was restored. It is conceivable that the great increase in resistance during the hypotonic period was caused by the occlusion of the gland lumens. Quantitative analysis of the Na, K, and Cl tissue concentrations indicated large loss of these ions during the hypotonic state. Presumably the epithelial cells in the hypotonic mucosae avoid bursting by rapidly losing large numbers of ions and, thus, results in cellular osmolarity close to that of the bathing fluids.

*Key words:* Gastric mucosa, frog, hypotonic solution, electrophysiology, microscopy

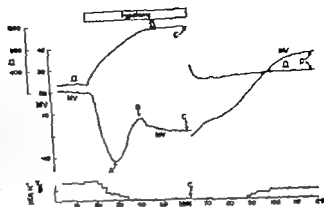
In a previously unpublished work (Sanders and Rehm) we found that by bathing the *in vitro* frog gastric mucosa with half-isotonic fluids the functions of the gastric mucosa were not markedly impaired. This raised the question as to whether the frog gastric mucosa could continue to function in very hypotonic bathing fluids. We determined the effect of hypotonic fluids which contained the same concentrations of  $\text{K}^+$ ,  $\text{Ca}^{++}$ ,  $\text{Mg}^{++}$  and  $\text{H}^+$  as the control solutions but had total osmotic pressure of from 25 to 30 mOsm (isotonic  $\sim 200$ ) and found that  $\text{H}^+$  secretion was abolished. We expected that the mucosal cells would be cytolized and no recovery of function would be possible, but instead, after restoration of the control isotonic fluids, the  $\text{H}^+$  rate recovered. We then studied the effects of hypotonic media on the electrical parameters, the ionic content and the histology of the gastric mucosa. The

whether the state in "chronically" decerebrate animals represents a recovery to normal capability of brain stem structures or whether supersensitivity develops. Whichever the case, the present study has demonstrated cerebellar projections to bulbar and neuron pools involved in emotional behaviour possibly the same which can be activated in intact animals or hypothalamic preparations.

This research has been sponsored by grants from the Swedish Medical Research Council (No 17640) and by grants from the Medical Faculty of Göteborg.

### References

- BARD, P. and M. B. MACHT. The behaviour of chronically decerebrate cats. In: *Cats: Form and function on the neurological basis of behaviour* Ed. G. E. W. Wolstenholme and C. M. O'Connor J. & A. Churchill Ltd., London, 1958, 55-75.
- HALL, W. K., H. W. MAGOUN and S. W. RANSOM. Localization within the cerebellum of reactions to its cerebellar stimulation. *J. comp. Neurol.* 1937 67 145-182.
- MARTNER, J., Cerebellar influences on autonomic mechanisms. An experimental study in the cat with special reference to the fastigial nucleus. *Acta physiol. scand.* 1973. Suppl. 425.
- MOMAKOW, C. von, *Die Lokalisation im Grosshirn und der Aufbau der Funktionen durch Leselekt.* J. P. Bergmann, Wiesbaden, 1914. 1 1033.
- ZANCHETTI, A. and A. ZOCORONI. Autonomic hypothalamic outbursts elicited by cerebellar stimulation. *J. Neurophysiol.* 1954 17 475-483.



1. Tissue resistance is shown for 1.3 cm<sup>2</sup> of mucosa, potential losses in mV (positive values are mucosal side positive), and H<sup>+</sup> secretory rate is one of the parameters. For further explanation see text.

at 4°C, rinsed again in nutrient, dehydrated in rising concentrations of ethyl alcohol, and finally embedded in Epon or Araldite.

Light microscopy was performed on one mucosa thick sections stained with toluidine blue. For the electronic analysis the point-counting method was used (Wambold *et al.*, 1969) with square lattices mounted in the eyepiece of the microscope. 5 sections from each of 5 animals were quantitated for each of the 3 states: isotonic, "hypotonic" and "reverts". For each animal the 5 sections were taken from different parts of the gastric mucosa. The relative volumes measured in this study were: epithelial cell volume, intercellular space ( $\mu$  mucus propria) and, except for the hypotonic condition, the lumen.

Electron microscopy was performed on thin (80 nm) ultramicrotome sections, which were contrasted with lead hydroxide and uranyl acetate.

## Results

### Fractional excretion

In preliminary experiments we found that if the hypotonic state (hypotonic solution on both sides) is continued for periods of over an hour before returning to isotonic solutions, the H<sup>+</sup> secretory rate either did not return or returned to only a small fraction of its original rate. However, there was good return of function after exposure of less than 1 h, and thus a period of about 30 min was used as the standard time of exposure to the hypotonic fluid (actual range was from 25 to 46 min).

Fig. 1 shows a typical experiment in which isotonic fluids were replaced with hypotonic ones on both sides. The H<sup>+</sup> secretory rate started decreasing within a few minutes and reached a zero rate in about 20 min. After the return of the isotonic fluids the H<sup>+</sup> rate returned in all of the 9 experiments (see Table II) after a latent period of about 15 min.

With hypotonic fluids the resistance increased and continued to increase over a period of about 15 min and in some experiments reached extraordinarily high levels. Frequent measurements of the resistance (every 10 s) revealed no transient decreases in the resistance, which could be expected if the cells had become damaged and leaky. Following the return to isotonic conditions the resistance decreased rapidly to a level near the original control level.

The PD during the hypotonic period showed an initial marked drop of about 30 mV (the mucosal side became negative by about 12 mV), and this was followed by a rebound

TABLE I Composition of incubation fluids (in mM).

	Secretory isotonic	Secretory hypotonic	Nutrient isotonic	Nutrient hypotonic
Solution No.	1	2	3	4
K <sup>+</sup>	4	4	4	4
Na <sup>+</sup>	100	7	102	7
Cl <sup>-</sup>	104	6	101	6
HCO <sub>3</sub> <sup>-</sup>	0	0	5	5
SO <sub>4</sub> <sup>2-</sup>	0	2.5	0.8	0.8
Ca <sup>++</sup>	0	0	1.0	1.0
Mg <sup>++</sup>	0	0	0.8	0.8
HPO <sub>4</sub> <sup>2-</sup>	0	0	1.0	1.0
Glucose	0	0	5.0	5.0
Sucrose	0	6	0	0
Osmolarity (mOsm)	208	25.5	220.6	30.6

Calculated on assumption osmotic coefficients are unity actual osmolarities  $\approx 3$  to  $8 \times 10^4$  m.

primary purpose of the present paper is to present the results of these findings. Previous reports of this work have been given (Sanders and Rehm 1968, Sanders, Shanbour and Rehm 1970).

### Methods

The experiments were performed on *Rana pyrena* with an *in vitro* method described in detail elsewhere (Rehm 1962, Rehm *et al.* 1963). Two pairs of electrodes were used, one pair for sending current through the mucosa and the other for measuring the PD. The resistance was determined as the ratio of the change in PD (after 0.5 s) per unit of applied current (Kidder and Rehm 1970, Noyes and Rehm 1970). The current was applied first in one direction and then in the other direction; there was no significant rectification. The solutions used in these experiments are given in Table I. The resistance values were corrected for the solution resistances on the two sides (36 ohm cm for the isotonic solutions and 250 ohm cm for the hypotonic solutions). After a control period, isotonic bathing media (No. 1 and 3, Table I) was placed in the chamber (No. 2 and 4 of Table I) on both sides of the mucosa for periods of about 30 min and then the fluid to the original controls (No. 1 and 3 of Table I). The original controls are referred to as "isotonic" and the experimental period as "hypotonic" and the return to isotonic conditions as "return". H<sub>2</sub>O<sub>2</sub>  $10^{-4}$  M was used in all nutrient solutions. The gas was 99% O<sub>2</sub> and 1% CO<sub>2</sub> so that we could use HCO<sub>3</sub><sup>-</sup> rather than 25 mM HCO<sub>3</sub><sup>-</sup> to maintain normal pH on the nutrient side. The use of 5 mM HCO<sub>3</sub><sup>-</sup> rather than 25 mM HCO<sub>3</sub><sup>-</sup> enabled us to achieve a tonicity around 10 mOsm. It should be pointed out that going from 95% O<sub>2</sub>-5% CO<sub>2</sub> to 99% O<sub>2</sub>-1% CO<sub>2</sub> and 5 mM HCO<sub>3</sub><sup>-</sup> to 11 mM HCO<sub>3</sub><sup>-</sup> will reduce the rate of respiration (Sanders, 1970 and Rehm 1973, Kidder 1974). However with 99% O<sub>2</sub>-1% CO<sub>2</sub> and an easily measured and relatively stable H<sub>2</sub> secretory rate the Na, K and Cl concentrations of the secretory and the hypotonic mucosae were determined by flame photometry and the Colloidal osmometry respectively by a method previously described (Shanbour, Davis and Rehm 1970). In preparing the tissue excised and loose mucus were removed by gently wiping the tissue with a glass rod. We did not remove the mucous coat because we previously found that attempts to do so result in loss of a substantial number of surface cells (Canoas and Rehm 1968).

### Histological methods

Fixation for morphological analyses was accomplished by adding a mixture of glutaraldehyde and formaldehyde (Peracchia and Müller 1972) to the secretory and nutrient solutions simultaneously. The final concentration of these compounds were 1 and 0.1% respectively. After fixing for 3 h the chamber the mucus was removed, rinsed in nutrient solution for 1 h, postfixed in an ice-cold 1% OsO<sub>4</sub> solution for 1 h.



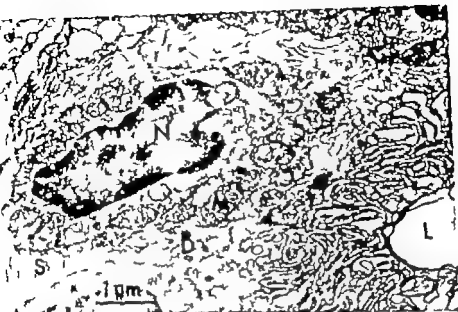


Fig. 2. Oxynic cell from frog gastric mucosa incubated in isotonic solution. An extensive network of secretory and lysosomal organelles from the cell uses the lumen (L) of the gastric tubule. Interdigitations are seen between the lateral cell membranes. The intercellular space (S) is up to about 0.3  $\mu$ m in this figure. D, desmosomes, M, mitochondrion, N, nucleus.  $\times 16,000$ .

than in the isotonic oxynic cells, and portions of the apical cytoplasm were frequently quite electron lucid. The intercellular spaces between neighboring oxynic cells had shrunk to roughly 20 nm—the same as between the surface epithelial cells. The shrinkage of the lamina propria appeared uniform. The distances in the sections between neighboring glands varied considerably but interglandular regions of  $\sim 25 \mu$ m were frequently seen extending from the base of the glands to the surface epithelial cells.

The ultrastructure of the epithelial cells after return to isotonic solutions (Fig. 4) was essentially the same as in control isotonic mucosae. The surface epithelial cells exhibited more or less flat apical surface with a few short microvilli. Numerous dense secretory granules were packed beneath this membrane, and the empty-looking regions of cytoplasm had disappeared. Intercellular spaces were still roughly 20 nm between the surface epithelial cells, but had returned to about 200 nm between the oxynic cells (as in the isotonic mucosae). The gland lumina appeared somewhat wider than in the isotonic mucosae.

By light microscopy the isotonic (Fig. 5) and the return mucosae were similar but the hypotonic mucosae (Fig. 6) exhibited swollen cells. This was most easily seen on the surface of the mucosae, where the epithelial cells were frequently seen to bulge into the gastric cavity. At deeper levels of the hypotonic mucosae most gland lumina were evidently obliterated by swollen oxynic cells. In some cases the regions where gland lumina could be expected were occupied by almost translucent material, which made it very difficult to determine whether the lumen was obliterated by a cell or not. Consequently it was not possible to

TABLE II Summary of physiological data from 9 return expts. Letters A through D refer to the intervals indicated on Fig. 1. A is for the nadir of the PD, B the peak of the rebound of the PD, C the time immediately before return to the isotonic condition, and D the time after a sec had been reached in the isotonic fluids.

	PD (mV)					Resistance ( $\Omega \text{ cm}^2$ )			H <sup>+</sup> Rate ( $\mu\text{Eq h}^{-1}$ )	
	Origin	At A	At B	At C	At D	Origin	At C	At D	Origin	At C
Average	24.4	-6.9	10.1	4.1	33.3	131	1 211	263	2.13	0
$\pm 3\text{S.D.}$	7.2	5.9	6.7	6.3	11.5	21	207.8	223	0.37	
Range	13	-16		-3	14	104	871	117	1.68	
	to	to	to	to	to	to	to	to	to	
	36	1	19	14	46	160	1 547	850	2.64	

and then a small decline. Following the return to the isotonic conditions the PD as showed a negative spike which was then followed by a marked increase in the PD, at the end of the experiment it was about 15 mV greater than its values in the control condition.

As shown previously (Spangler and Rehm 1968) the time constants for diffusion of molecules via the diffusion barriers separating the bathing fluids from the mucosa layer is about a minute or less. Thus the mucosal cell layer is exposed to the full range of the hypotonic fluids within a matter of a few minutes.

Table II presents a summary of 9 expts. In every experiment the H<sup>+</sup> secretory rate decreased to zero during the hypotonic state and was reestablished following the return to isotonic conditions. The resistance showed an approximately 10-fold increase in the hypotonic state and recovered to a level which was slightly higher than the original level. The PD (at D) in 6 out of 9 expts. was about 20 mV higher than the original level in 3 it was lower.

### Morphological results

The ultrastructure of the normal frog gastric mucosa has previously been described by several authors (Geuze 1971 Soder 1965 Forte, G. M. Limlomwongse and Forte, 1969), and our findings on the isotonic mucosae did not differ significantly from earlier results. Since much recent interest is devoted to transintercellular pathways, it should be noted that the intercellular space between neighboring surface epithelial cells was generally in the range of 20 nm. However between adjacent oxyntic cells, the intercellular space was much wider up to a few hundred nm (Fig. 2). This space was very irregularly formed, limited apically by the terminal bar region. Desmosomes were frequently found between surface epithelial cells, but were not so common between oxyntic cells.

The hypotonic mucosae exhibited a marked swelling of the cells. The apical surface of the surface epithelial cells was convex, being semispherical with an absence of microvilli. In some instances the plasma membrane was broken. In the unbroken cells the secretory granules were mixed with regions of very low electron density and flocculent or weblike material. The oxyntic cells were also swollen, and like the surface epithelial cells abnormalities were more in the apical portions of the cells (Fig. 3). In most cases the gland lumen was completely obliterated. The ground cytoplasm and nucleus were generally lower density

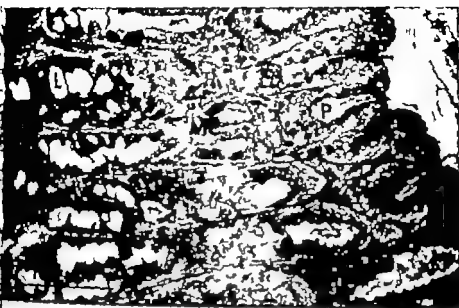


Fig. 4. Survey of gastric mucosa from return stomach. This mucosa looks essentially similar to the "normal" mucosae, but the lower gland lamina appear somewhat deleted. L, lumen of gastric gland; NC, mucous neck cell, P gastric pit.  $\times 300$ .

hypotonic mucosae was  $14.1$  (S.D.  $\pm 0.8$ ) while for the isotonic mucosae it was  $11.3$  (S.D.  $\pm 0.8$ ). Assuming no change of dry weight in the hypotonic mucosae then on the basis of the above figures it follows that the volume for the hypotonic mucosae was 1.25 the volume of the isotonic controls. From a comparison of the actual volumes with the assumptions that the increase in volume was uniform (*i.e.* the same for the epithelial cell portion as for the rest of the mucosa) it follows from our morphometric data that the epithelial cell volume of the hypotonic mucosae ( $0.73 \div 1.25 = 0.94$ ) was about twice that of the controls ( $0.48 \div 1.0 = 0.48$ ), and the interstitial fluid volume was about 60% of that of the controls.

Table IV also shows the K, Na and Cl content of the mucosae. Three other hypotonic experiments were performed in which the Na and Cl concentrations were slightly different in the hypotonic secretory solution than in the nutrient solution. The results of these latter experiments were essentially the same as those in Table IV.

Other workers report lower values for Na and higher values for K in the *in vitro* frog gastric mucosae (*e.g.* Davenport 1963, Davenport and Alzamora 1962). However these workers removed the adherent mucous coat and since we did not remove this coat our values would be expected to be different.

#### Discussion

Many more questions have been raised by the results of the foregoing experiments than could possibly be answered within the scope of a single paper. It is clear that the frog gastric mucosa can recover after exposure of the mucosa to very hypotonic fluids for periods of

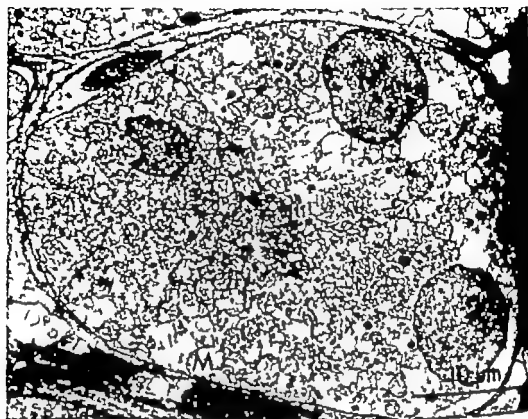


Fig. 3. Cross section of gland tubule from "hypotonic" mucosa. At this level of the mucosa the gland is made up of oxyntic cells. These cells are swollen to the extent that the lumen is totally obliterated. The former place is indicated in the center of the tubule (L). Mitochondria (M) and cell nuclei (N) are visible but the ground cytoplasm appears vacuolated or dissolved. The intercellular spaces are much more patent than in the isotonic mucosae. In comparison with these also the nucleus seems to be a lost mass of electron density  $\times 3000$ .

obtain meaningful morphometric values for the relative volumes of the lumen in the hypotonic mucosa. For the subsequent calculations of water content (see p. 12) it was of interest to estimate the volume of the epithelial cell layer and lamina propria in relation to the total volume of the chambered tissue flap. In the controls, the epithelial cell layer plus lamina propria occupied roughly 56% (S.D. 15.9) of the total tissue volume, whereas the muscularis mucosae plus the portion of the submucosa included in the preparation occupied 44%. In the hypotonic and in the returns the corresponding values for the epithelial cell layer plus lamina propria were 51% (S.D. 8.4) and 52% (S.D. 4.1), respectively.

The relative epithelial cell volume + the volume of the gland and pit lumina (see Table I) were significantly larger in the hypotonic mucosae than in the isotonic ( $p < 0.005$ ). Moreover the relative epithelial cell volume was significantly larger in the returns than in the isotonic ( $p < 0.01$ ). Also the relative lumen volume was significantly larger in the returns than in the isotonic ( $p < 0.05$ ).

#### *Water and electrolyte content of isotonic and hypotonic mucosae*

The water content of the hypotonic mucosae was significantly greater than that of the isotonic controls (see Table IV). Thus, the average ww/dw (wet weight/dry weight) for the

TABLE III. Morphometric data on frog gastric mucosa. Values are given in per cent of total volume of epithelial cells and lamina propria (means  $\pm$  S.D.,  $n=5$ ).

	Isotonic	Hypotonic	Return
epithelial cells	48.4 $\pm$ 9.1	—	63.0 $\pm$ 3.2
lumen and pit lumen	3.5 $\pm$ 0.7	—	11.4 $\pm$ 6.0
epithelial cells + lamina	52.0 $\pm$ 9.5	75.4 $\pm$ 2.8	74.4 $\pm$ 5.5
lamina propria	48.0 $\pm$ 9.5	24.6 $\pm$ 2.8	25.6 $\pm$ 5.5

calculated value of 80 down to about 25 mOsm/L. Although we are not able to arrive at precise values for the loss of ions from the cells the data in Table IV are compatible with the idea that there is sufficient loss of electrolyte from the cells to bring their osmolarity down to the level of the hypotonic fluid. Since it is generally assumed that mammalian cells would burst if a sizable hydrostatic pressure develops, it seems reasonable to believe that a substantial hydrostatic pressure never develops between the cells and the ambient media. Our tentative explanation is that the increase in water content of the cells and the loss of electrolytes result in a cellular osmolarity equal to that of the hypotonic media.

#### *Changes of the resistance during the hypotonic state*

The resistance increases seen in the hypotonic experiments are in general larger than we have seen with other experimental conditions. In this section we present a possible explanation for the very large increases in resistance which is based on our findings and upon other findings presented in preliminary form (Sanders and Rehm 1968, Sanders, Shanbour and Rehm 1970). This explanation is no more than a tentative working hypothesis. First the question arises as to whether the very large increase in resistance is due to the high resistance of the hypotonic fluids. Using either the secretory or the nutrient hypotonic fluids in the chamber without a mucosa we found a resistance of about 250 ohm  $\text{cm}^2$  which is about 210 ohm  $\text{cm}^2$  greater than the resistance with isotonic solutions in the chamber in the absence of a mucosa. Calculations of the resistance of the gastric mucosa if it was replaced with a hypotonic solution reveal that the resistance would be about 90 ohm  $\text{cm}^2$ . Thus, whether we subtract 210 ohm  $\text{cm}^2$  or 160 ohm  $\text{cm}^2$  (210-50) makes little difference from the point of view of explaining the very large increase in resistance with the hypotonic media. Con-

TABLE IV. Water, K, Na and  $\text{Cl}^-$  contents in isotonic and hypotonic mucosae (means  $\pm$  S.D.,  $n=4$ ). WW = wet weight, DW = dry weight.

Condition	WW/ DW	K mEq/kg $\text{H}_2\text{O}$	K mEq/kg DW	Na mEq/kg $\text{H}_2\text{O}$	Na mEq/kg DW	$\text{Cl}^-$ mEq/kg $\text{H}_2\text{O}$	$\text{Cl}^-$ mEq/kg DW
Isotonic	11.3 $\pm$ 0.6	32.6 $\pm$ 5.8	336 $\pm$ 66	116.8 $\pm$ 4.0	1202 $\pm$ 100	92.6 $\pm$ 3.0	931 $\pm$ 84
Hypotonic	14.1 0.8	18.4 $\pm$ 3.4	241 $\pm$ 30	14.4 $\pm$ 1.8	189 $\pm$ 28	9.1 $\pm$ 1.6	120 $\pm$ 14



Fig. 5



Fig. 6

Fig. 5. Light micrograph of frog gastric mucosa incubated in isotonic solutions. The lamina (L) of the tubules are clearly visible in the basal half of the mucosa. They open into the gastric pits (P). Sorta propria (MM) muscularis mucosae. 40

Fig. 6. Light micrograph of hypotonic mucosa. The cells of the gastric glands are greatly swollen, much less stained than in the isotonic mucosae. Clon eggs are seen in the gland tubules, but often difficult to decide whether these are gland lumina (L) or swollen projections from the axons. Due to the swelling the lamina propria (LP) appears greatly reduced. The surface epithelial cells are so bulge into the gastric cavity. Also the cells of the muscularis mucosae (MM) are swollen. 40.

30 mm. The morphological findings indicate that the gastric cells swell in hypotonic media and that the interstitial fluid decreases. When the increase in the total volume of the mucosa in the hypotonic solutions is taken into account the cells swell about 2 times (or more) of water to the cells and assessing a dry weight to cells of 30% the water content of the cells would increase by about 2.5 times. So if no solutes exited from the cells the expected osmolarity of the cells (with a 2.5 increase in water content) would be about 80 mOsm/L. The osmolarity of the hypotonic media is about 25 mOsm/L so for the cells to have the same osmolarity as the hypotonic media there must be sufficient loss of solutes to bring it

1. JENN, W. S., T. L. DAVIS, C. CHANDLER, E. GOSMANN, J. and A. BARNERLAHR, Frog gastric mucosae bathed in  $\text{Cl}^-$ -free solutions. *Amer. J. Physiol.* 1963, 204, 233-42.
2. JENN, W. S. and W. S. REISS, Effect of hypotonic solutions on *in vitro* frog gastric mucosa. *Biophys. J.* 1966, 2, WFE.
3. JENN, W. S., V. B. HAYNE, JR. and W. S. REISS, Normal  $\text{H}^+$  rates in frog stomach in absence of exogenous  $\text{CO}_2$  and  $\text{HCO}_3^-$  on the pH stat method. *Amer. J. Physiol.* 1973, 225, 1311-1315.
4. JENN, W. S., L. L. SUMMOUR and W. S. REISS, Resistance changes of *in vitro* frog gastric mucosa bathed in very hypotonic fluids. *Biophys. Soc. Abstr.* 1970, 10, 113.
5. JENN, W. S. Fine structure of the stimulated oxyntic cells. *Fed. Proc.* 1965, 24, 1360-1367.
6. SUMMOUR, L. L., T. L. DAVIS and W. S. REISS, Electrolyte content of the cornel stomach of the bullfrog. *Proc. Soc. exp. Biol. (N.Y.)* 1970, 135, 11-13.
7. SUMMOUR, L. L. and W. S. REISS, Potential responses of nutrient membranes of frog stomach to step changes in external  $\text{K}^+$  and  $\text{Cl}^-$  concentrations. *Biophys. J.* 1968, 8, 1211-1227.
8. JENN, W. S., W. S. LUM, H. R. GILLES and F. A. HERR, Correlated morphometric and biochemical studies on the liver cell. I. Morphometric model, stereological methods, and partial morphometric data for six liver. *J. cell Biol.* 1968, 42, 68-91.

sequently if the mucosa was replaced with hypotonic fluids and there were no gross considerations we would not expect such a large increase in resistance. Furthermore, if there were no changes in the geometry of the cells and the cytoplasmic resistance increased 17 fold this should make little difference in the magnitude of the resistance because the resistance across epithelia is primarily a function of the resistance of the limiting membranes of the cells and not of the cytoplasm. Therefore, we have to look at changes in the geometry. Our tentative hypothesis is that the surface cells (at least under the hypotonic conditions) have a very high resistance and that the low resistance seen normally with isotonic fluid (100 to 200 ohms  $\text{cm}^2$ ) is due to a low resistance of the tubular cells. Our major postulate is that the very high resistances seen with hypotonic fluids is due to a partial or almost complete occlusion of the lumen of the tubular cells. The big range of the resistance values from Table II might then reflect the degree of occlusion of the lumina. Complete occlusion would result in an essentially infinite resistance via the lumen. In a previous electron microscopic study the total surface area of the secretory membranes (including gastric pits, pits, and microvilli etc.) of the *in vitro* frog gastric mucosa was found to be about 210 times the macroscopic area and that of the oxyntic cells alone 190 times (Helander *et al.* 1970). Hence complete occlusion of the lumen would result in a reduction of roughly 90% of the surface area (190/210 = 100) available for the passage of current. Assuming that the secretory membrane surfaces all have the same resistance in terms of ohms for a  $\text{cm}^2$  of actual surface the resistance would be expected to increase by about 10-fold which is approximately what we found experimentally.

Supported by grants from the Swedish Medical Research Council (project no. 12X 2298) and the National Institutes of Health (project no. 2R01 EY00456) and the National Science Foundation (project no. C 72-02508).

## References

- CANOSA, C. and W. S. REHM, Microelectrode studies of dog's gastric mucosa. *Biophys. J.* 1968, 8, 41.
- DAVENPORT H. W. Sodium space and acid secretion in frog gastric mucosa. *Amer. J. Physiol.* 1962, 203, 213-216.
- DAVENPORT H. W. and F. ALZAMORA, Sodium, potassium, chloride, and water in frog gastric mucosa. *Amer. J. Physiol.* 1962, 202, 711-715.
- FORTE, G. M., L. LINDLÖW-WONN and J. G. FORTE, The development of intracellular membranes coincident with the appearance of HCl secretion in oxyntic cells of the metamorphosing tadpole. *J. Cell Biol.* 1969, 4, 709.
- GRUZI, J. J. Light and electron microscope observations on the gastric mucosa of the frog (*Rana esculenta*). *Z. Zellforsch.* 1971, 117, 87-102.
- HELANDER, H. F., S. S. SANDERS, W. S. REHM and B. I. HIRSHENOWITZ, Quantitative aspects of gastric physiology. In G. Sachs and E. Heinz (eds.) *Gastric secretion*, pp. 69-88. Academic Press, New York.
- KIDDER, G. W. and C. W. MONTGOMERY,  $\text{CO}_2$  diffusion into frog gastric mucosa as rate-limiting factor in acid secretion. *Amer. J. Physiol.* 1974, 227, 300-304.
- KIDDER, G. W. and W. S. REHM, A model for the long time-constant transient voltage response to electrical stimulation of epithelial tissues. *Biophys. J.* 1970, 10, 215-236.
- NOTES, D. H. and W. S. REHM, Voltage responses of the frog gastric mucosa to direct current. *Am. J. Physiol.* 1970, 219, 184-192.
- PERACCHIA, C. and B. S. MITTLER, New glutaraldehyde fixation procedures. *J. Ultrastruct. Res.* 1970, 35, 57-64.
- REHM, W. S., Acid secretion, resistance, short-circuit current and voltage-clamping in frog's mucosa. *Amer. J. Physiol.* 1962, 203, 63-72.



at a normal or slightly elevated level, *i.e.* autoregulation of the blood flow (for reference see Thürow 1964). In the second major theory suggested by Thurau and his colleagues (Thurau 1964, Thurau and Schniermann 1965, Schniermann *et al.* 1970) the sodium concentration (or the load) at the macula densa segment of the nephron was thought to be a determinant of the renin release from the juxtaglomerular cells, and thereby of the production of Angiotensin II a determinant of the afferent vascular tone. This latter hypothesis has in recent years been the objective of several investigations. The majority of these would seem to support the original suggestion at least when the sodium load was increased to super-normal levels, whereas a decreased load, on the contrary rarely produces the expected decrease in afferent vascular tone. (See Schniermann *et al.* 1973.)

In the present paper the control of the afferent vascular tone as predicted by the myogenic hypothesis has been examined. The transmural pressure has then been reduced by increasing the renal interstitial pressure. The vascular reactions with respect to the resistance pattern within the superficial cortical vasculature has been analyzed via the study of the blood flow and the hydrostatic pressures within the different segments of the cortical vascular tree. The interstitial pressure was continuously monitored via about 50  $\mu$ m thick PVC-catheters put under the renal capsule and placed into the subcapsular space.

### Material and Methods

All experiments were carried out on male Sprague-Dawley rats weighing between 250-340 g. The animals were anaesthetized with Inactin® (120 mg/kg) and placed on a nerve-controlled heating pad. The left kidney was exposed via flank incision and suspended into a Lucite cup. The different parameters were analyzed in different ways according to:

**Microcirculatory studies.** For the study of intravascular and extracellular pressures, a nerve-controlled pressure device according to Wiederholzer (1964) as modified by Isaguerre (1970) was used. The system was connected to sharpened glass capillaries with tip diameter of about 4  $\mu$ m. Glomerular capillary pressure was measured indirectly using the stop-flow technique according to Gertz (1966) (the technique consisted essentially of first injecting into an early proximal tubular segment until the filtration fluid ceased. The stop-flow pressure then established as measured with a second cannula inserted proximally to the first cannula. The glomerular capillary pressure was calculated by adding this pressure to the colloid osmotic pressure of the systemic plasma, the latter as calculated from data on the protein content in the plasma using the formulas given by Landis and Pappenheimer (1963). Effort was made to inject the early part of the proximal tubules. Randomly punctured tubules will have a stop flow pressure of 1-3 mm Hg below the pressure in the most early part of the nephron (as investigated in some control experiments). Only the latter result will corroborate with the directly punctured glomerular blood vessels (a) on the kidney surface. The afferent arteriole pressure was measured as the pressure within the renal vessels easily identified on the kidney surface. The peritubular capillary pressure was obtained from randomly punctured small peritubular capillaries. Occasionally superficial small veins are identifiable allowing a more complete evaluation of the pressure profile of the vascular network. Identification of the structure punctured is made by injection of Leucine green or Evans blue through the same pipette that used for the pressure measurements.

The glomerular filtration both with respect to single superficial nephron filtration as well as to total renal filtration was analyzed using 125-Iodithalamate as the test substance. The amount of activity injected amounted to 100  $\mu$ Ci/h or less. The single glomerular filtration rate was investigated on the same tubule as those used for the determination of the stop-flow. For this purpose a small droplet of about 5 tubule lengths was injected, immediately followed by the suction of tubular fluid at a rate to keep the oil droplet in fixed position. The time of sampling was 2-3 min. During the sampling the intratubular pressure was continuously monitored by the pressure pipette inserted proximally to the sampling pipette. Only the samples in which this pressure was normal or slightly decreased were accepted. For the determination of the 125-I activity the sampling pipettes were put into tubes and counted in a gamma-detector. Please

## Effect of Elevated Interstitial Pressure on the Renal Cortical Hemodynamics

By

Ö. KÄLLSKOG and M. WOLGAST

Received 18 March 1975

---

### Abstract

KÄLLSKOG Ö and M. WOLGAST *Effect of elevated interstitial pressure on the renal cortical hemodynamics* Acta physiol. scand. 1975. 95. 364-372.

The influence of renal interstitial pressure on the resistance pattern within the superficial cortical zone has been investigated from determinations of 1) the glomerular blood flow with a modified microtechnique and 2) the intravascular hydrostatic pressures.

Interstitial pressure was monitored with a 50  $\mu$ m PVC-catheter placed into the subcapsular space. Two conditions were analyzed: a) elevation of urethral pressure to 70 mm Hg and b) stress to 10-15 mm Hg. Both conditions produced an increase in the interstitial pressure from 1-2 to about 5 mm Hg as well as a decreased hilar lymph flow and protein flow of about the same size. Vascular reactions were different, however. Urethral stress (but not the stress of a single nephron) produced a decreased resistance in the afferent arterioles with concomitant increase in the pressures in glomerular capillaries, and the peritubular capillary network. In contrast, stress stress produced small changes in the parameters studied but for the obvious rise in the peritubular capillary pressure.

The results suggest that factors other than the interstitial pressure are governing the afferent arterioles, the tubular wall tension might be one of these factors.

---

One of the more important characteristics of the renal blood flow refers to the ability of the kidneys to regulate their own blood supply when the perfusion pressure varies from about 70 mm Hg to 200 mm Hg, i.e. autoregulation. This autoregulative response has been found to operate in intact kidneys in situ as well as in isolated kidneys perfused with whole blood or plasma (e.g. Thurau and Kramer 1959 a, 1959 b, Ochswald 1956, Waugh and Waugh and Shanks 1960, Hinshaw 1964, Thurau and Henne 1964). The causative factors, however, underlying this response remain largely unidentified. Two main hypotheses have been suggested. In the classical myogenic hypothesis the transmural pressure difference in the afferent arteriole is primarily thought to be the determinant of the vascular tone, thereby the blood flow in the sense that an increased transmural pressure would lead to arteriolar constriction and a decreased pressure to a vasodilatation. An increased blood pressure would then increase the transmural pressure causing an increased afferent arteriole resistance. This would result in a restitution of an initially increased blood flow back to

mal or slightly elevated level, *i.e.* autoregulation of the blood flow (for reference see Low 1964). In the second major theory suggested by Thurau and his colleagues (Thurau & Thurau and Schoenmann 1965 Schoenmann *et al.* 1970) the sodium concentration (the load) of the macula densa segment of the nephron was thought to be a determinant for renal release from the juxtaglomerular cells, and thereby via the production of Angiotensin II, a determinant of the afferent vascular tone. This latter hypothesis has in recent years been the objective of several investigations. The majority of these would seem to support the original suggestion — at least when the sodium load was increased to super-normal levels, whereas a decreased load, on the contrary rarely produces the expected decrease in afferent vascular tone. (See Schoenmann *et al.* 1973)

In the present paper the control of the afferent vascular tone as predicted by the myogenic hypothesis has been examined. The transmural pressure has then been reduced by increasing renal interstitial pressure. The vascular reactions with respect to the resistance pattern when the superficial cortical vasculature has been analyzed via the study of the blood flow and the hydrostatic pressures within the different segments of the cortical vascular tree. The interstitial pressure was continuously monitored via about 50  $\mu$ m thick PVC-catheters inserted under the renal capsule and placed into the subcapsular space.

### Material and Methods

All experiments were carried out on male Sprague-Dawley rats weighing between 280–340 g. The animals were anesthetized with ketalar (120 mg/kg) and placed on a servocontrolled heating pad. The left kidney was exposed via flank incision and suspended into a Lucite cup. The different parameters were analyzed in different series according to:

**Microvascular studies.** For the study of intratubular and intertubular pressures, a servo controlled pressure sensor device according to Wiedersheim (1964) as modified by Irtigheima (1970) was used. The system is connected to sharpened glass capillaries with an tip diameter of about 4  $\mu$ m. Glomerular capillary pressure was measured indirectly using the stop-flow technique according to Gerls (1966). In this technique coloured mineral oil is first injected into an early proximal tubular segment until the interstitium had ceased. The stop-flow pressure then established was measured with a second cannula inserted proximally to the first cannula. The glomerular capillary pressure is calculated by adding this pressure to the colourless osmotic pressure of the systemic plasma, the latter is calculated from data on the protein concentration in the plasma using the formulae given by Landis and Peppenhauer (1963). Effort is made to reduce the early part of the proximal tubules. Randomly punctured tubules will have a stop flow pressure of 1–3 mm Hg below the pressure in the most early part of the nephron (as investigated in some control experiments). Only the latter result will corroborate with the directly punctured glomeruli found occasionally in 3% on the kidney surface. The efferent arteriole pressure was measured as the pressure within the renal vessels easily identified on the kidney surface. The peritubular capillary pressure was obtained from randomly punctured small peritubular capillaries. Occasionally superficial small veins are identified showing more complete evaluation of the pressure profile of the vascular network. Identification of the structures punctured was made by injection of Lissamine green or Evans blue through the same papilla as that used for the pressure measurements.

The glomerular filtration both with respect to single superficial nephron filtration as well as to total renal filtration was analyzed using 125-Iothalamate as the test substance. The amount of activity injected amounted to 100  $\mu$ Ci/20  $\mu$ l or less. The single glomerular filtration rate was investigated on the same tubules as those used for the determination of the stop-flow. For this purpose a small droplet of about 5 tubular lengths is injected, immediately followed by an injection of tubular fluid at a rate to keep the oil droplet at a fixed position. The time of sampling is 2–5 min. During the sampling the intratubular pressure was continuously monitored by the pressure papette inserted proximally to the sampling papette. Only those samplings in which the pressure is normal or slightly decreased was accepted. For the determination of the 125-I activity the sampling papettes were put into tubes and counted in a gamma-detector. Plasma

TABLE I Cardiac output, total renal blood flow, superficial and juxtamedullary glomerular blood flow during control antidiuresis and during the elevation of the ureteral pressure (upper part) and during the elevation of the renal venous pressure (lower part). From the table it could be concluded that the conditions induced will be accompanied by essentially no changes in the conditions studied

Experimental condition	Cardiac output ml/min 100 g	Experimental kidney ml/min 100 g	Control kidney ml/min 100 g	Superficial glomeruli ml/min 100 g	Juxtamedullary glomeruli ml/min 100 g
Control, n=13 P=70 mm Hg	27.2±5.1 27.3±8.6	1.91±0.16 1.76±0.50	1.92±0.44 1.90±0.79	99.0±79.4 93.1±28.0	69.0±15.3 73.3±45.6
Control, n=9 P=11.0±0.8 mm Hg	32.7±5.0 6.7±4.0	2.30±0.36 1.95±0.10	2.41±0.40 2.15±0.43	129.5±20.0 131.2±21.7	110.5±36.2 104.8±32.6

activity was determined from blood samples withdrawn during the middle of the sampling period. Estimation of the total filtration, urine was sampled in 10–15 min periods.

The interstitial pressure was measured as the pressure within the subcapsular interstitial space with the same pressure measuring device, but the glass capillary was replaced by a thin PVC-catheter of diameter of less than 50  $\mu$ m. The catheter was introduced through a hole in the renal capsule as in the subcapsular space about 1 mm from the hole. The hole was then sealed with a silicon rubber pad (Källskog and Wolgast 1973). These measurements were performed separately after the end of the micropuncture studies since the registration of two pressures (afferent capillary and interstitial) would interfere with each other.

The ureteral blood flow conditions were investigated with the microsphere technique using  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  labelled 3M microspheres (3M Co., St Paul, Minn. USA). The spheres were first with ultrasonic agitation and then separated by sedimentation in large glass cylinders at deceleration (Källskog *et al.* 1972). The size range of these spheres could then be reduced to  $\pm 2.6 \mu$ m and with no aggregate among the injected spheres. 400 000 of each type of spheres, suspended in rat plasma, were injected approximately 70 s through a catheter inserted into the right carotid artery just above the aortic valves. During the injection blood was continuously sampled from the femoral artery at a rate of 0.6 ml/min. The blood flow of the kidneys and single glomeruli were analyzed according to Bankir *et al.* 1973.

The aortic lymph was collected from small PE-catheters inserted into the aortic lymph vessel. A lymph flow sampling condition was considered to occur when the lymph vessel was somewhat proximal to the catheter's tip. The flow rate was determined from the length of the fluid column above glass capillaries (microscope 5.20 and 100  $\mu$ m, Drummond Scientific Co., USA). The protein content in the plasma and lymph was analyzed according to Lowry (1951).

Two experimental conditions were studied: 1) elevated ureteral pressure to 20 mm Hg and 2) renal venous pressure to 11–15 mm Hg. The latter condition was produced by constriction of the vein with a thin thread placed around the vessel. The venous pressure was monitored via a steel cannula inserted distally to the structure.

## Results

The results from the blood flow study during elevated ureteral pressure and elevated renal venous pressure, respectively are shown in Table I. The cardiac output was about 30 ml/min 100 g rat in all the conditions studied. The total renal blood flow was close to 2 ml/min 100 g rat and showed no differences between the experimental and the control kidneys. Since the micropuncture data primarily refer to the superficial structures, the blood flow conditions in these superficial glomeruli are of special interest. In these glomeruli the blood flow is relatively large—estimated at about 100 ml/min per 100 g rat. The juxtamedullary

TABLE II. Peritubular "Stop-flow" pressure and hydrostatic pressures within the superficial cortical vasculature, proximal tubules, subcapsular interstitial space together with the data on single nephron and total kidney filtration and on the urine production during control and/or ureteral and/or venous occlusion (upper panel) or renal venous (lower panel) stasis. For explanation see text.

Experimental conditions	Stop-flow pressure mm Hg	Glomerular pressure mm Hg	Capillary pressure mm Hg	Tubular pressure mm Hg	Total renal filtration ml/min 100 g	Single nephron filtration ml/min 100 g	Urine flow $\mu$ l/min	Interstitial pressure mm Hg
control	6 $32.8 \pm 1.4$	13 $13.0 \pm 1.4$	11 $11.1 \pm 1.4$	13 $13.3 \pm 1.7$	0.32 $\pm 0.04$	14 $14.3 \pm 1.7$	3.1 $\pm 2.6$	1.3 $\pm 0.7$
20 mm Hg	40 $33 \pm 2.5$	18 $18.9 \pm 1.8$	16 $16.3 \pm 2.0$	22 $22.3 \pm 1.8$	0.35 $\pm 0.10$	16 $16.2 \pm 1.5$	3.6 $\pm 4.4$	4.0 $\pm 1.6$
control	8 $35.3 \pm 3.2$	15 $15.3 \pm 1.7$	12 $12.5 \pm 2.1$	14 $14.8 \pm 1.4$	0.49 $\pm 0.08$	21 $21.4 \pm 1.3$	6.6 $\pm 2.4$	1.0 $\pm 0.3$
14 mm Hg	33 $33.9 \pm 4.6$	20 $20.6 \pm 3.6$	17 $17.6 \pm 2.8$	19 $19.5 \pm 4.2$	0.34 $\pm 0.05$	13 $13.7 \pm 2.9$	8.2 $\pm 0.8$	5.0 $\pm 2.5$

lowers, here depicted for reference purposes, showed slightly lower values. From Table I it could seem that the blood flow in all the structures studied will remain essentially unchanged during both the induced ureteral and venous stasis.

The micropuncture data from 6 rats subjected to ureteral pressure elevation and 8 rats with venous pressure elevation are depicted in Table II. Single nephron and total glomerular filtration rates have been studied in 6 and 4 expts. in the two groups, respectively. The values obtained from one individual experiment were derived from 3-6 single determinations of pressures or determinations of a single nephron filtration. The scatter of these variables in the individual experiment was negligible: the pressures in the peritubular capillary network, for instance, did not differ from each other more than 1-2 mm Hg. The differences from one experiment to the other were also very slight which seems clear from the table. The interstitial pressures shown to the right in Table II were not measured in connection with the other measurements, but estimated after the completion of the micropuncture studies.

Table II is utilized for the description of the pressure profile within the cortical superficial vascular network shown in Fig. 1. The control values are in accordance with our own previous data as well as those of others (Andreucci *et al.* 1971, Brenner *et al.* 1972, Falchuk *et al.* 1971 a, 1971 b) and requires therefore only few comments. The single nephron filtration rates are in these series relatively large (see Glebech, Symposium on Renal Micropuncture Techniques (1972)), but in accordance with the likewise high superficial glomerular blood flow found in the study. As the hematocrit in the rats used is slightly below 50%, the plasma flow will amount to about 50 ml/min, this should then mean a filtration fraction of somewhat less than 30%, which would seem reasonable. It is somewhat puzzling, however, that the filtration rates in the study with venous stasis were even higher. This is most likely due to the higher blood flow as well as the somewhat higher glomerular capillary hydrostatic pressure found in this series. The reason for this phenomenon remains unexplained. However, the manipulation of the renal vein with the division of the vessel from the artery and the application of a thread for clamping may be a contributing cause. Otherwise the rats were of identical age and were treated in the same way.

Elevation of the ureteral and venous pressures to such an extent that the same increase in

TABLE 1 Cardiac output, total renal blood flow, superficial and juxtamedullary glomerular blood flow during control antidiuretics and during the elevation of the ureteral pressure (upper panel) and during the elevation of the renal venous pressure (lower panel). From the table it could seem that the conditions induced will be accompanied by essentially no changes in the circulatory parameters studied

Experimental condition	Cardiac output ml/min 100 g	Experimental kidney ml/min 100 g	Control kidney ml/min 100 g	Superficial glomeruli nl/min 100 g	Juxtamedullary glomeruli nl/min 100 g	Weight g
Control, $n=13$	$27.2 \pm 5.1$	$1.91 \pm 0.16$	$1.92 \pm 0.44$	$99.0 \pm 29.4$	$69.0 \pm 13.3$	$232 \pm 4$
$P=20$ mm Hg	$27.3 \pm 8.6$	$1.76 \pm 0.50$	$1.90 \pm 0.79$	$93.1 \pm 28.0$	$71.5 \pm 43.9$	
Control, $n=9$	$32.7 \pm 5.0$	$2.30 \pm 0.36$	$2.41 \pm 0.40$	$129.5 \pm 20.0$	$110.5 \pm 36.2$	$262 \pm 11$
$P=11.0 \pm 0.8$ mm Hg	$26.7 \pm 4.0$	$1.95 \pm 0.10$	$2.15 \pm 0.43$	$131.2 \pm 21.7$	$104.8 \pm 32.6$	

activity was determined from blood samples withdrawn during the middle of the sampling period. For the estimation of the total filtration urine was sampled in 10–15 min periods.

The *interstitial fluid pressure* was measured as the pressure within the subcapsular interstitial space using the same pressure measuring device, but the glass capillary was replaced by a thin PVC-catheter with a diameter of less than  $50 \mu\text{m}$ . The catheter was introduced through a hole in the renal capsule and placed in the subcapsular space about 1 mm from the hole. The hole was then sealed with a silicon rubber compound (Kalliskog and Wolgast 1973). These measurements were performed separately after the completion of the micropuncture studies since the registration of two pressures (e.g. capillary and interstitial) was found to interfere with each other.

The *ureteral blood flow* conditions were investigated with the microsphere technique using  $^{141}\text{Ce}$  and  $^{85}\text{Sr}$  labelled 3M microspheres (3M Co. St Paul Minn. USA). The spheres are first treated with ultrasonic agitation and then separated by sedimentation in large glass cylinders as described previously (Kalliskog *et al.* 1972). The size range of these spheres could then be reduced to  $\pm 2.63 \mu\text{m}$  ( $\pm 5.0$  and with no aggregate among the injected spheres. 400 000 of each type of spheres, suspended in 0.5 ml of rat plasma, were injected in approximately 20 s through a catheter inserted into the right carotid artery with its tip placed just above the aortic valves. During the injection blood was continuously withdrawn from the femoral artery at a rate of 0.6 ml/min. The blood flow of the kidneys and single glomeruli was analyzed as according to Bankir *et al.* 1973.

The *renal hilar lymph* was collected from small PE-catheters inserted into a hilar lymph vessel. Accurate lymph flow sampling conditions were considered to occur when the lymph vessel was somewhat collapsed proximal to the catheter's tip. The flow rate was determined from the length of the fluid column in constant bore glass capillaries (microcaps 5.20 and 100  $\mu\text{l}$ , Drummond Scientific Co., USA). The protein concentration in the plasma and lymph was analyzed according to Lowry (1951).

Two experimental conditions were studied: 1) elevated ureteral pressure to 20 mm Hg and 2) elevated renal venous pressure to 11–15 mm Hg. The latter condition was produced by constriction of the renal vein with a thin thread placed around the vessel; the venous pressure was monitored in a steel cannula inserted distally in the structure.

## Results

The results from the blood flow study during elevated ureteral pressure and elevated venous pressure, respectively, are shown in Table 1. The cardiac output was about 30 ml/min in 100 g rat in all the conditions studied. The total renal blood flow was close to 2 ml/min in 100 g rat and showed no differences between the experimental and the control kidneys. Since the micropuncture data primarily refer to the superficial structures, the blood flow conditions in these superficial glomeruli are of special interest. In these glomeruli the blood flow is relatively large—estimated at about 100 nl/min per 100 g rat. The juxtamedullary

11. Data on the renal hilar lymph flow and albumin flux after the induction of either elevated ureteral (upper panel) or elevated venous pressure. The table demonstrated that the conditions studied were accompanied with clear and stable rise in the hilar lymph flow and protein flux, whereas the lymph protein concentration shows a barely significant decrease. The fractional change corresponds to the increase in interstitial pressure found under the same conditions, which could be predicted if the interstitial pressure monitored was representative for the whole of the renal interstitium.

units)	Lymph flow $\mu\text{l}/\text{min}$	Protein con- centration g	Protein flux $\mu\text{g}/\text{min}$
<i>n</i> , 7	$0.46 \pm 0.24$	$1.7 \pm 0.2$	$8 \pm 5$
1 mm Hg	$1.11 \pm 0.82$	$1.4 \pm 0.3$	$17 \pm 16$
renal change	$2.3 \pm 0.7$	$0.8 \pm 0.4^*$	$1.9 \pm 0.6$
<i>n</i> , 4	$1.8 \pm 0.9$	$2.7 \pm 1.8$	$50 \pm 32$
8-15 mm Hg	$3.8 \pm 1.4$	$2.3 \pm 0.3$	$86 \pm 49$
renal change	$2.2 \pm 0.4$	$0.9 \pm 0.2^*$	$1.9 \pm 0.7$

\* significant change ( $P < 0.01$ ).

† significant change ( $P < 0.10$ ).

corpuscle was decreased in this series. At control conditions 0.4% of the total filtration filtered in the urine against the figure of 0.8% for the venous pressure elevation in spite that the glomerular filtration had decreased.

In these two series the interstitial pressure was raised about 3 times greater than the control rate. This ought to be followed by an increased lymph flow. For this reason the lymph flow and the lymph protein flux were investigated in connection with the study of the intrarenal blood flow distribution. The results are depicted in Table III. The table shows that during the conditions studied the lymph flow was increased, i.e. in accordance with the stated prediction, both from a qualitative and also approximately from a quantitative point of view. The lymph flow increased with 285% and 330% during ureteral and venous clamping, respectively. The increased lymph flow was in both series accompanied by a slight decrease in the lymph protein concentration; the lymph protein flow was raised to 270% and 290% during ureteral and venous clamps, respectively.

## Discussion

### Methodological

Where the pressure data is concerned some comments with respect to the measurements of the renal interstitial pressure should be made. The measurement of subcapsular interstitial pressure (hydrostatic and colloid-osmotic) was introduced by Wunderlich *et al.* in 1971. It was then argued that the subcapsular space from an anatomical point of view is a part of the renal interstitium. It was shown to be in free and rapid communication with the rest of the interstitium as indicated by the rapid appearance (about 20 min) of radioactive albumin in the hilar lymph after a single injection through a small PVC catheter inserted into the subcapsular space. The time of the albumin transport equals the time of passage from the peritubular blood stream to the hilar lymph (Wolgast *et al.* 1973).

During cold artificial perfusion with solutions of different compositions the interstitial

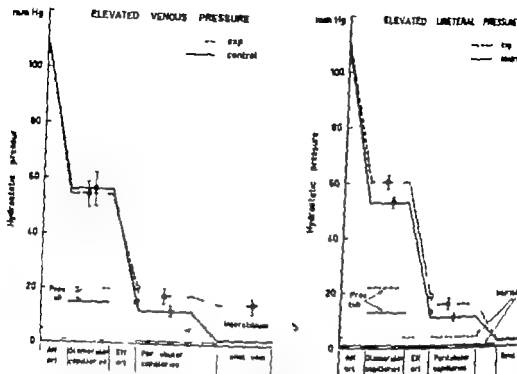


Fig. 1 Hydrostatic pressures within the superficial cortical vasculature, the proximal tubules and subcapsular interstitium (mean values  $\pm$  S.D.) during control antidiuretic conditions and the elevated ureteral pressure (right panel) and during control antidiuretic conditions and the elevation of the renal venous pressure (left panel). As the single superficial glomerular blood flow remained unchanged during the course studied, the figure suggests that ureteral stasis will be accompanied with a relaxation of the afferent arteriole with a concomitant pressure increase in the glomerular capillaries as well as in the peritubular capillary system. During venous stasis the glomerular capillary pressure was unchanged.

the interstitial pressure would occur produced clearly different patterns in the two groups studied. After the ureteral pressure elevation the glomerular capillary hydrostatic pressure was significantly raised. This combined with the fact that the blood flow was unaltered would mean a relaxation of the afferent arteriole. The driving force for glomerular filtration in the proximal end of the glomerulum (stop flow pressure minus proximal tubular pressure) in this series was estimated at 19.5 mm Hg at control conditions, decreasing to 18.0 mm Hg during the increased ureteral pressure, i.e. a negligible change. The single glomerular filtration rate showed a small but insignificant increase. The total filtration showed the same appearance with a slight change from 0.32 ml/min/100 g. The urine production showed an increase of about the same size which then means that the tubular fraction reabsorption also remained unchanged.

The phenomena described are restricted to ureteral stasis i.e. stasis of all the nephrons. Blockade of one single nephron (by distal oil blocks) was not accompanied by these reactions.

During venous pressure elevation the pressure drop over the glomerular capillary branches showed a clear decrease from 20.7 to 14.4 mm Hg. This was accompanied by a decrease in the single nephron filtration rate of about the same magnitude. The total filtration rate showed the same pattern with a decrease from 0.49 ml/min/100 g. It should again be noted that the figure of 0.49 ml/min/100 g is larger than the corresponding value found during the control condition in the series with ureteral clamping. Fraction



ring venous stasis the tubular pressure increased as a consequence of the increased interstitial pressure. The tubular transmural pressure however will be different—increased during ureteral and decreased during venous stasis. If this increased tubular wall tension could be a determinant of the afferent arteriolar tone remains unclear.

The experimental conditions analyzed in the present work have previously been the object of an intensive study. Most of the earlier investigations deal with determinations of the intrarenal vascular resistance as analyzed from data on the total renal blood flow and the glomerular filtration rate. In the majority of these studies a more or less pronounced increase in the total renal blood flow have been noticed during the elevation of ureteral pressure (Gibson 1964, Selkurt 1963, Thurman 1964). The subject has more recently been studied by (M *et al.* (1968) in which increased tubular pressure was induced by a urinary obstruction and by a large urine flow. In these studies the increase in the total renal blood flow on changing the ureteral pressure was attributed to a decreased afferent arteriolar tone. This in turn was thought to be due to an increased interstitial pressure producing a decreased transmural pressure difference. During elevation of the renal venous pressure only slight changes in the total vascular resistance will occur (Haddy *et al.* 1958).

Where micropuncture studies are concerned a clear increase in the peritubular capillary and the proximal tubular pressures has been found both as a consequence of the elevated arterial and venous pressures (Gottschalk and Muller 1956, Allison *et al.* 1972). These investigations also showed that the peritubular capillary pressure will raise in parallel with the renal venous pressure but finally when the latter pressure equals the normal pressure within the peritubular network, i.e. at about 10 mm Hg. Likewise Brenner *et al.* (1972) showed that tubular stop-flow in an isolated proximal tubular segment will increase with an increasing ureteral pressure, a phenomenon compatible with the increased glomerular capillary hydrostatic pressure as discussed here. To summarize, it can be stated that even though a more detailed description now is available for the reactions that occur during the actual experimental conditions, an acceptable explanation is still lacking.

## References

- ANDERSON, V. E., J. HERRERA-ACOSTA, F. C. Rector, Jr and D. W. SELDIN. Effective glomerular filtration pressure and single nephron filtration rate during hydropnea, elevated ureteral pressure, and some venous exposures with isotonic saline. *J. clin. Invest.* 1971 50 2236-2254.
- ALLISON, M. E. M., E. M. LEMMAN and C. W. GOTTSCHALK. Hydrostatic pressure in the rat kidney. *Amer. J. Physiol.* 1972 223 975-983.
- BALNER, L. N., FARMAN and J. P. OGDEN. Radioactive microsphere distribution and single glomerular blood flow in the normal rabbit kidney. *Physiol. Arch. ges. Physiol.* 1972. 342 111-123.
- BALNER, L. N., J. L. TROY and T. M. DADGAR. Pressures in cortical structures of the rat kidney. *Amer. J. Physiol.* 1972 222 246-251.
- FALCK, K. H. and R. W. BEALMER. Hydrostatic pressures in peritubular capillaries and tubules in the rat kidney. *Amer. J. Physiol.* 1971 220 1422-1426.
- FALCK, K. H., B. M. BALNER, M. TADOKORO and R. W. BEALMER. Osmotic and hydrostatic pressures in peritubular capillaries and fluid reabsorption by proximal tubule. *Amer. J. Physiol.* 1971 b. 220 1427-1433.
- FELLOW, B. Description of the myogenic hypothesis. *Circulat. Res.* 1964 14-15 279-287 Suppl. I.
- FELDMAN, L. A., ENGBERG, O., KALLEROD and M. WOLGAST. Renal transplantation in the rat. IV. Cortical pressure gradients measured in vivo and during isolated perfusion with reference to renal function after transplantation. *Scand. J. clin. Lab. Invest.* 1975 35 463-472.

pressure was found to be inversely related to the colloid-osmotic pressure of the perfused, reaching values up to 30 mm Hg during the perfusion with colloid-free sol (Frödin *et al* 1975). Negative interstitial pressures could also be obtained when sol with very high colloid osmotic pressures (10% albumin) and low perfusion rates were used. Furthermore, the increase in perfusion pressure during colloid-free perfusion compared with that during 5% albumin perfusion on these cold "passive kidneys" could be related to the increase in the interstitial pressure. This phenomenon could be predicted to occur if the measured interstitial pressure was representative for the whole of the renal interstitium. Arguments referred to thus suggest that the present measurements are valid. Previous studies in which so-called needle-pressure was used as the interstitial pressure would be unlikely due to the simple fact that these pressures equal the proximal tubular pressure and thereby are considerably higher than the distal tubular pressure (about 5 mm Hg).

### *Physiological*

The conditions studied have in common that the interstitial pressure was raised to the same extent. A further increase in the ureteral and the venous pressures (the latter has been made in the pilot series preceding the present study), showed a further increase in interstitial pressure. However even if the interstitial pressure changed to the same extent the vascular reactions were completely different. This strongly indicates that the interstitial pressure *per se* is not a determinant of the afferent arteriolar tone.

During *venous pressure elevation* the primary event would seem to be an increase in peritubular capillary pressure. The increase is, however, less pronounced than the increase in the venous pressure. The pressure drop between the peritubular capillaries and the renal vein will thereby vanish. The localization of the resistance responsible for the steep pressure drop between the peritubular capillaries and the renal vein during normal conditions remains unclear.

The glomerular capillary pressure in this series is not changed, which then means that the pressure drop and also the resistance over the efferent arterioles was decreased—since the blood flow was unchanged. In contrast afferent arteriolar tone remained essentially unchanged. The proximal tubular pressure was elevated producing a decreased driving force for glomerular filtration and consequently a concomitant decrease in the amount of fluid filtered. The cause for the pressure elevation in the tubules is obviously due to the rise in interstitial pressure compressing the tubules, *i.e.* a passive increase.

In the series on *ureteral stasis* the *primary* change is the increase in the tubular pressure. The possibility now exists that the tubules compress the interstitium between the tubular wall and the stiff renal capsula. This would, however, only lead to a transient increase in interstitial pressure and only a transiently increased renal lymph flow. This explanation for the increased interstitial pressure can therefore be ruled out. Instead the interstitial pressure rise is produced by the increased peritubular capillary pressure which in turn is due to the decreased afferent arteriolar tone. The outflow resistance, which in the case of the venous pressure elevation was decreased, will under these conditions be somewhat increased.

The two conditions analyzed now share another change in the parameters studied, *i.e.* the increased proximal tubular pressure. During ureteral stasis the increase is obvious where

## On the Possible Involvement of Sulphomucopolysaccharides in the Storage of Catecholamines within the Central Nervous System

by

CHRIS PYCOCK, ELEONORA BLASCHKE, ULLA BERQVIST and BÖRJE UVNÄS

Received 3 April 1975

### Abstract

PYCOCK, C., E. BLASCHKE, U. BERQVIST and B. UVNÄS. *On the possible involvement of sulphomucopolysaccharides in the storage of catecholamines within the central nervous system.* Acta physiol. scand. 1975. 95. 373-382.

Rats treated at birth with 6-OH DA were sacrificed as adults age 24 h after an injection of H-NA. Synaptosomes were isolated from the cerebral cortex, the hypothalamus, the pineal gland and the cerebellum. The similar distribution pattern of H-NA and  $^{35}$ S on gradient centrifugation of the synaptosome preparations, the similar effect of 6-OH DA on the uptake of  $^3$ H-NA into slices of brain tissue in contrast to their  $^{35}$ S content as well as the identification of chondroitin and heparan sulphate in the synaptosome fractions are observations which indicate a possible function of SMPSs in the storage of NA in subnuclear terminals.

It has been proposed that a sulphomucopolysaccharide-protein (SMPS-prot) complex may be involved in the binding of catecholamines (CA) within vesicles of various tissues (Uvnäs 1973). However it seems to be still a matter of controversial opinions; some workers find evidence for such an involvement (Fillion, Nosal and Uvnäs 1971 Åberg *et al.* 1972 Margolis and Margolis 1973 Margolis, Jaanus and Margolis 1973) while others have reported and interpreted results to the contrary (De Prada, von Berlepsch and Pletscher 1972). The main tissue that has been subjected to investigation appears to be the adrenal medulla (Fillion, Nosal and Uvnäs 1971 Margolis and Margolis 1973 Margolis, Jaanus and Margolis 1973) while little reference is made to the storage of CA within the central nervous system. The distribution of sulphomucopolysaccharides (SMPSs) among subcellular fractions has been investigated only in preparations from the whole brain (Vos *et al.* 1966 Karhunen and Okada 1971 Bacchawat *et al.* 1972).

This report presents the results of such a study utilizing synaptosomes—pinched-off

- GERTZ, K. H. J. A. MANGOOS, G. BRAUN, and H. D. PAGEL, Pressure in the glomerular capillaries in rat kidney and its relation to arterial blood pressure. *Pflügers Arch. ges. Physiol.* 1966. 262. 369-75.
- GILMORE, J. P. Renal vascular resistance during elevated ureteral pressure. *Circulat. Res.* 1964. 1: 148-152. Suppl. 1.
- GOTTSCHEALK, C. W. and M. MYLLE, Micropuncture study of pressures in proximal tubules and peritubular capillaries of the rat kidney and their relation to ureteral and venous pressures. *Amer. J. Physiol.* 1953. 185. 430-439.
- HADDY, F. J. J. SCOTT, M. FLESHMAN and D. EMANUEL, Effect of change in renal venous pressure on renal vascular resistance, urine and lymph flow rates. *Amer. J. Physiol.* 1952. 199. 111-119.
- HINSHAW, L. B., Mechanism of renal autoregulation. Role of tissue pressure and description of a factor hypothesis. *Circulat. Res.* 1964. 14-15. 170-182. Suppl. 1.
- INTAGLIETTA, M. R. F. PAWULA and W. R. TOMPKINS, Pressure measurements in the mammalian microvasculature. *Microvasc. Res.* 1970. 2. 212-220.
- KÄLLSKOG, Ö. and M. WOLGAST, Driving forces over the peritubular capillary membrane in the rat during acid diuresis and saline expansion. *Acta physiol. scand.* 1973. 89. 116-124.
- KÄLLSKOG, Ö. H. R. ULFENDAHN and M. WOLGAST, Single glomerular blood flow as measured with carbonized <sup>141</sup>Ce labelled microspheres. *Acta physiol. scand.* 1972. 85. 408-413.
- KIL, F. J. KRISHNA and E. LOVNING, Role of autoregulation in maintaining glomerular filtration rate under flow. *Acta physiol. scand.* 1969. 76. 24-39.
- LANDIS, E. M. and J. R. P. PENNHEIMER, Exchange of substances through the capillary wall. *Handb. Physiology. Circulation II*. Waverly Press, USA. 1963. 961-1034.
- LOWRY, O. H. N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the phenol reagent. *J. Biol. Chem.* 1951. 193. 263-275.
- OCHTAWDT, B., Zur Selbststeuerung des Nierenkreislaufes. *Pflügers Arch. ges. Physiol.* 1956. 262. 29.
- Renal Micropuncture Techniques. A Symposium. Ed. G. H. Giebisch, *Yale J. Biol. Med.* 1972. 43.
- SCHNEIDERMAN, J. A. E. G. PERSSON and B. ÅGERUP, Tubuloglomerular feedback. Nonlinear relationship between glomerular hydrostatic pressure and loop of henle perfusion rate. *J. clin. Invest.* 1973. 51. 869.
- SCHNEIDERMAN, J. F. S. WRIGHT, J. M. DAVIS, W. V. S. ACKELBERG and G. GRILL, Regulation of renal nephron filtration rate by tubulo-glomerular feedback. *Pflügers Arch. ges. Physiol.* 1971. 338. 147-175.
- SELKURT, E. E., Effect of ureteral blockade on renal blood flow and urinary concentrating ability. *J. Physiol.* 1963. 205. 386-392.
- THURAU, K., Renal hemodynamics. *Amer. J. Med.* 1964. 36. 698-719.
- THURAU, K. and G. HERGENROTHER, Die transnephronale Druckdifferenz der Widerstandsgefäße als Parameter der Widerstandsregulation der Niere. *Pflügers Arch. ges. Physiol.* 1964. 279. 156-177.
- THURAU, K. and K. KRAMER, Die Reaktionsweise der glatten Muskulatur der Nierengefäße auf Dehnung und ihre Bedeutung für die Autoregulation des Nierenkreislaufes. *Pflügers Arch. ges. Physiol.* 1959. 268. 188-203.
- THURAU, K. and K. KRAMER, Weitere Untersuchungen zur myogenen Natur der Autoregulation des Nierenkreislaufes. *Pflügers Arch. ges. Physiol.* 1959. 269. 77-93.
- THURAU, K. and J. SCHNEIDERMAN, Die Natriumkonzentration an den Maculadensa Zellen als regulierender Faktor für das Glomerulumsfiltrat (Mikropunctureversuche). *Klin. Wochenschr.* 1963. 43. 410-413.
- WAUGH, W. H., Myogenic nature of a autoregulation of renal flow in the absence of blood cells. *Circulat. Res.* 1958. 6. 363-372.
- WAUGH, W. H. and R. G. SHANKS, Cause of genuine autoregulation of the renal circulation. *Circulat. Res.* 1960. 8. 871-882.
- WUNDERLICH, C. A., J. W. WOODS, Y. S. KIRK and R. F. RUSSELL, Pulsatile pressures in the microcirculation of frog's mesentery. *Amer. J. Physiol.* 1964. 207. 171-176.
- WOLGAST, M. H. R. ULFENDAHN, Ö. KÄLLSKOG, H. RASMUSSEN, J. L. A. KINS and G. G. PINTER, Protein distribution in kidney. *Proteins of the biological fluids*—21st colloquium. Ed. H. Peeters. Pergamon Press, Oxford and New York. 1973. 413-417.
- WUNDERLICH, P. E. PERSSON, J. SCHNEIDERMAN, H. R. ULFENDAHN and M. WOLGAST, Hydrostatic pressure in the subcapsular interstitial space of rat and dog kidney. *Pflügers Arch. ges. Physiol.* 1971. 328. 31.

## On the Possible Involvement of Sulphomucopolysaccharides in the Storage of Catecholamines within the Central Nervous System

By

CARL FYCOCK, ELEONORA BLANCHER, ULLA BERGQVIST and BORJE UVNÄS

Received 3 April 1975

### Abstract

FYCOCK, C., E. BLANCHER, U. BERGQVIST and B. UVNÄS. *On the possible involvement of sulphomucopolysaccharides in the storage of catecholamines within the central nervous system* Acta physiol. scand. 1975, 95, 373-382.

Kits treated at birth with 6-OH DA were sacrificed as adults age 24 h after an injection of <sup>3</sup>H-NA. Synaptosomes were isolated from the cerebral cortex, the hypothalamus, the post-midbrain region and the cerebellum. The similar distribution pattern of <sup>3</sup>H-NA and <sup>35</sup>S on gradient centrifugation of the synaptosomes preparations, the similar effect of 6-OH DA on the uptake of <sup>3</sup>H-NA into slices of brain tissue in culture and on their <sup>35</sup>S content as well as the identification of chondroitin and heparan sulphate in the synaptosomal fractions are observations which indicate possible functions of SMPs in the storage of NA in presynaptic terminals.

It has been proposed that a sulphomucopolysaccharide-protein (SMPs-pro) complex may be involved in the binding of catecholamines (CA) within vesicles of various tissues (Uvnäs 1973). However it seems to be still a matter of controversial opinions, some workers finding evidence for such an involvement (Fillon, Nosal and Uvnäs 1971 Åberg *et al.* 1972, Margolis and Margolis 1973, Margolis, Jaanus and Margolis 1973) while others have reported and interpreted results to the contrary (Da Prada, von Borel and Pletscher 1972). The main tissue that has been subjected to investigation appears to be the adrenal medulla (Fillon, Nosal and Uvnäs 1971 Margolis and Margolis 1973 Margolis, Jaanus and Margolis 1973) while little reference is made to the storage of CA within the central nervous system. The distribution of sulphomucopolysaccharides (SMPs) among subcellular fractions has been investigated only in preparations from the whole brain (Vos *et al.* 1969 Karhama and Okada 1971 Bacchawat *et al.* 1972).

This report presents the results of such a study utilizing synaptosomes—pinched-off

nerve terminals produced by homogenisation of brain tissue—as functional units from discrete areas of the rat brain (for reviews, see Whittaker 1969). This has been combined with the effect of chemical denervation with 6-hydroxydopamine (6-OH DA) to investigate the possible relationship between CA and  $^{35}\text{S}$ -labelled compounds associated with the monoamine-storing sites. An attempt to characterize and identify sulphur-containing compounds has been made.

## Methods and Materials

Albino rats (Sprague Dawley) were used in the experiments. For chemical denervation of the neurotoxic compound 6-OH DA (3–100 mg/kg, dissolved in saline containing 0.2 mg/ml ascorbic acid) was administered subcutaneously to newborn rats in a volume of 0.05 ml. The initial dose was within 4 h after birth and the subsequent 2 doses on the following days at 4 h intervals. Control received a similar injection sequence of saline. All animals were sacrificed by cervical dislocation under light chloroform anaesthesia, at the age of 10–14 weeks. Radioactive sodium sulphate ( $\text{Na}_2^{35}\text{S}$ , free, Kjeltek, Norway) was administered subcutaneously in a dose of 8 mCi/kg 24 h before sacrifice.

### Preparation of synaptosomes

Several brain regions (cerebral cortex, cerebellum, hypothalamus and pons) were dissected out and homogenized in 10 volumes of 0.25 M sucrose using a glass homogenizer with teflon pestle at a speed of about 1000 rpm. The homogenates were centrifuged at 600  $g$  for 10 min and the supernatant layer used as the crude synaptosome preparation for incubation studies.

### In vitro incubation of synaptosomes

Aliquots (100  $\mu\text{l}$ ) of the supernatant layer were incubated in Krebs-Ringer bicarbonate buffer lacking 6-hydroxydopamine (6-OH DA). In order to determine the amount of amine taken up (see Jonsson *et al.* 1974). After 5 min incubation period at 37°C (Sachs and Jonsson 1972) the reaction was terminated by the addition of 8 ml cold Krebs-Ringer buffer and the mixture centrifuged at 1000  $g$  for 10 min to produce a synaptosomal pellet. The pellet was dissolved in 1 ml 1 N NaOH, then the ethanol-toluene emulsification solution was added and the H and  $^{35}\text{S}$  radioactivity determined. The uptake at 0°C, which can be considered as extraneuronal uptake, has been subtracted from the uptake obtained at +37°C (= active uptake).

### Glycerol centrifugation

In other experiments a pre-incubated synaptosomal fraction was layered on a linear continuous density gradient (3.5 ml) ranging from 0.3–1.6 M sucrose, as previously described (Jonsson *et al.* 1974). Following centrifugation at 75 000  $g$  for 2 h the tubes were pierced at the bottom and about 25  $\mu\text{l}$  of H and  $^{35}\text{S}$  was determined.

### Lysis of synaptosomes

In some experiments the synaptosomal pellet after labelling with H-NA, was taken and resuspended in 1 ml distilled water to lyse the subcellular particles. After allowing the suspension to stand at room temperature for 1 h, the tubes were centrifuged at 50 000  $g$  for 15 min. The amounts of radioactivity in H and NA present in the pellet or supernatant component were determined.

### Identification of $^{35}\text{S}$ -labelled compounds

**(i) Isolation and purification of SMPs.** Crude synaptosomal pellets, some of them lysed, were dried *in vacuo* overnight, extracted with chloroform-methanol and the extract examined with thin layer chromatography for presence of sulphatides. The extracted pellet was subjected to protein hydrolysis with pepsin according to Olsson *et al.* (1968), followed by precipitation of the residual protein with trichloroethanol according to Sjögren and an Robertson (1967). The supernatant layer was examined by thin layer chromatography on Sephadex G-50 according to Margolis and Margolis (1970) for presence of glycoproteins. Aliquots of all isolation steps were taken for measurement of  $^{35}\text{S}$  radioactivity.

**Separation and determination of SAMPs.** 2 different separation procedures are carried out on the 15% polyacrylamide microelectrophoresis on cellulose acetate strips according to Weisler (1964) and fractionation on cellulose columns according to Svajcar and de Robertson (1967), both before and after treatment with chondroitinase AC. Pieces of the electrophoretic strips, resp. aliquots of the column eluates are taken for measurement of  $^{35}\text{S}$ -radioactivity. The strips are dissolved in acetic acid which was then counted. 10 ml Instagel (Packard) was added to the samples and the  $^{35}\text{S}$  radioactivity measured.

#### Measurement of radioactivity

A Packard liquid scintillation spectrometer 3375 was used. All results were corrected for quenching by the external standard method. In case of simultaneous labelling with  $^3\text{H}$  and  $^{35}\text{S}$  double-channel analysis was applied and corrections made for quenching and for spillover between the channels.

## Results

### $^3\text{H}$ -NA uptake and synaptosomal $^{35}\text{S}$ content

Control rats and rats treated at birth with 6-OH DA were sacrificed in the adult age, 24 h following an injection of  $^{35}\text{S}$ -labelled sodium sulphate. Pretreatment at birth caused a marked decrease in the *in vitro* uptake of  $^3\text{H}$ -NA into synaptosomes from the cerebral cortex, being about 20% of control values, while the uptake in the hypothalamic synaptosomes is somewhat less affected, 70% of control (Fig. 1). However  $^3\text{H}$ -NA uptake in synaptosomes isolated from the pons-medulla region showed a considerable increase, being about 100% of control value (Fig. 1). A comparison of the  $^{35}\text{S}$  content of each synaptosomal preparation showed also a decrease in the cortical and hypothalamic regions isolated from rats treated with 6-OH DA at birth to the order of 65-70% of control values, whereas no change was detected in the pons-medulla region (Fig. 1).

### Gradient centrifugation studies

In sucrose gradient centrifugation studies, the  $^3\text{H}$ -NA-labelled synaptosomes from control animals showed similar equilibrium characteristics as the distribution of  $^{35}\text{S}$  on the gradient

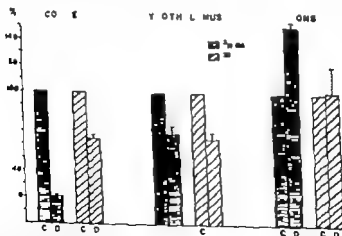


Fig. 1 *In vitro* uptake of  $^3\text{H}$ -NA and radioactive  $^{35}\text{S}$ -content of synaptosomes isolated from various regions of the brain of control rats (C) and denervated animals (D) treated at birth with 6-OH DA. The animals were sacrificed at the adult age. Each column represents the mean  $\pm$  S.E. of 3 determinations and the values are expressed as percentage of control.

nerve terminals produced by homogenisation of brain tissue—as functional C units from discrete areas of the rat brain (for reviews, see Whittaker 1969). This has been combined with the effect of chemical denervation with 5-hydroxydopamine (DA) to investigate the possible relationship between CA and  $^{35}\text{S}$ -labelled compounds associated with the monoamine-storing sites. An attempt to characterize and identify purine-containing compounds has been made.

### Methods and Materials

Albino rats (Sprague Dawley) were used in the experiments. For chemical denervation of the neurotoxic compound 6-OH DA (3–100 mg/kg, dissolved in saline containing 0.2 mg/ml ascorbic acid) was administered subcutaneously to newborn rats in a volume of 0.05 ml. The initial dose was within 4 h after birth and the subsequent 2 doses on the following days at 4 h intervals. Control received a similar injection sequence of saline. All animals were sacrificed by cervical dislocation under light chloroform anaesthesia, at the age of 10–14 weeks. Radioactive sodium sulphate ( $\text{Na}_2^{35}\text{SO}_4$ , free, Kjeltak, Norway) was administered subcutaneously in a dose of 8 mCi/kg 4 h before sacrifice.

#### *Preparation of synaptosomes*

Several brain regions (cerebral cortex, cerebellum, hypothalamus and pons) were dissected out and homogenized in 10 volumes of 0.25 M sucrose using a glass homogenizer with a teflon pestle at a speed of about 1000 rpm. The homogenates were centrifuged at 600  $g$  for 10 min and the supernatant layer used as the crude synaptosome preparation for incubation studies.

#### *In vitro incubation of synaptosomes*

Aliquots (100  $\mu\text{l}$ ) of the supernatant layer were incubated in Krebs-Ringer bicarbonate to which 11-noradrenaline ( $^{3\text{H}}$  NA) was added in order to determine the amount of amine taken up (see Jonsson *et al.* 1974). After 3 min incubation period at 37°C (Sachs and Jonsson 1972) the reaction was terminated by the addition of 9 ml cold Krebs-Ringer buffer and the mixture centrifuged at 600  $g$  for 10 min to produce a synaptosomal pellet. The pellet was dissolved in 1 ml 1 N N OH, then ethanol-toluene scintillation solution was added, and the 11 and  $^{35}\text{S}$  radioactivity determined. Uptake at 0°C, which can be considered as extraneuronal uptake, has been subtracted from the values obtained at +37°C (= active uptake).

#### *Gradient centrifugation*

In other experiments a pre-incubated synaptosomal fraction was layered on a linear continuous density gradient (3.5 ml) ranging from 0.3–1.6 M sucrose, as previously described (Jonsson *et al.* 1974). Following centrifugation at 75 000  $g$  for 2 h the fractions were pierced from the bottom and about 0.5 ml collected. After addition of 10 ml toluene phosphor Triton X 100 mixture (4:1) the radioactivity of 11 and  $^{35}\text{S}$  was determined.

#### *Lysis of synaptosomes*

In some experiments the synaptosomal pellet after labelling with 11 NA, was taken up in 1 ml distilled water to lyse the subcellular particles. After allowing the suspension to stand at room temperature for 1 h, the tubes were centrifuged at 50 000  $g$  for 15 min. The amount of radioactivity of 11 NA present in the pellet or supernatant component were determined.

#### *Identification of $^{35}\text{S}$ -labelled compounds*

a) *Isolation and purification of SNAPSs.* Crude synaptosomal pellets, some of them lysed, were incubated overnight, extracted with chloroform-methanol and the extract examined with thin layer chromatography for presence of sulphatides. The extracted pellet was subjected to protein hydrolysis according to Olsson *et al.* (1968), followed by precipitation of the residual protein with trichloroacetic acid. From the dialyzed supernatant layer the SNAPS were precipitated with 5% potassium ethanol according to Svecjar and Albertsson (1967). The supernatant layer was examined by thin layer chromatography on Sephadex G 50 according to Margolis and Margolis (1970) for presence of glycosaminoglycans. Aliquots of all isolation steps were taken for measurement of  $^{35}\text{S}$  radioactivity.



Percentage release of radioactive sulphur and tritium into soluble fractions of synaptosomes from different brain regions following lysis in distilled water. Each figure represents the mean  $\pm$  S.E. of 4 experiments.

Region	Released	
	$^{35}\text{S}$	$^3\text{H-NA}$
Cortex	$17.3 \pm 3.3$	$79.1 \pm 3.5$
Hypothalamus	$21.7 \pm 6.9$	$83.6 \pm 2.9$
Cerebellum	$21.7 \pm 4.4$	$81.9 \pm 4.4$
Pons	$14.5 \pm 3.2$	$90.6 \pm 3.6$

2). Synaptosomes from cortex, cerebellum and hypothalamus showed a peak eluting at 0.9–1.2 M sucrose. The hypothalamus also yielded a smaller secondary peak at 0.7 M sucrose. A similar distribution pattern was observed for  $^{35}\text{S}$ .

#### Release of synaptosomes

Following lysis, the synaptosomes from the cortex, hypothalamus and cerebellum lost out 20% of their  $^{35}\text{S}$  content which appeared in the supernatant layer. The percentage soluble  $^{35}\text{S}$  in the pons was slightly lower about 15% (Table I).

#### Subcellular patterns of $^{35}\text{S}$ -incorporation into synaptosomes

Radioactive sulphur was found to be incorporated in several types of compounds isolated using fractionation of the crude synaptosomal pellet (Table II). The cortex, hypothalamus and cerebellum show a very similar pattern. roughly one third is represented by lipid-bound  $^{35}\text{S}$ , one third by sulphomucopolysaccharide-bound and about one third was recovered in a supernatant layer after SMPS precipitation. 3–4% remained in the residual protein precipitate, probably partly due to adsorption and partly to incomplete breakdown of the SMPS-protein linkage. In the synaptosomal pellet isolated from the pons the prevalent form of  $^{35}\text{S}$  was found incorporated in the lipid fraction.

#### Isotopic characterization of the $^{35}\text{S}$ -labelled compounds

The SMPSs isolated from synaptosomes of different brain regions showed essentially the same electrophoretic pattern. On the barium acetate electrophoretogram 2 maxima of  $^{35}\text{S}$  radioactivity were detected. The Rf of one of them corresponded to the Rf of the reference dermatan sulphate, the other peak lay between the reference compounds of dermatan sulphate and chondroitin sulphate, thus pointing to the possible presence of a dermatan sulphate-chondroitin sulphate hybrid. The result of the separation of the SMPSs on cellulose columns is represented in Fig. 3. On fractionation of the SMPSs on cellulose columns

\* Distribution of radioactivity in synaptosomes (labelled *in vitro* with H-NA) from a) cerebral cortex, b) hypothalamus and c) cerebellum on continuous sucrose density gradients ranging from 0.3–1.6 M sucrose. The gradients were centrifuged at 75 000 g for 120 min. Animals were injected 24 h previously with  $^{35}\text{SO}_4$ . (—) depicts tritium distribution and (---)  $^{35}\text{S}$  distribution. Representative diagrams have been chosen from 3–5 experiments.

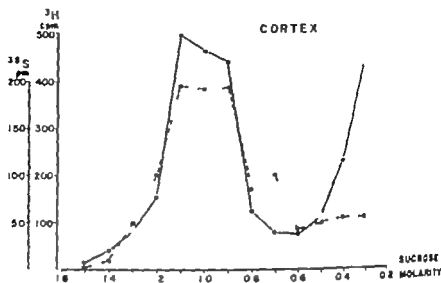


Fig. 2a.

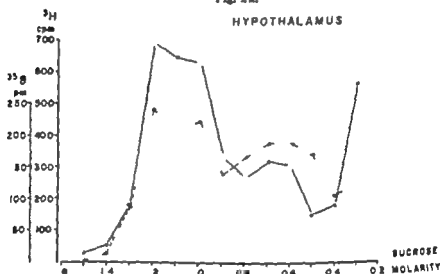


Fig. 2b.

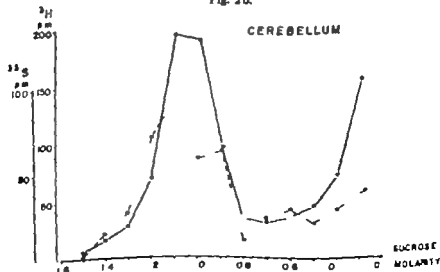


Fig. 2c.

Percentage release of radioactive sulphur and tritium into soluble fractions of synaptosomes from different brain regions following lysis in distilled water. Each figure represents the mean  $\pm$  S.E. of 4 experiments.

Regions	Released	
	$^3\text{H}$	H-NA
Cerebral cortex	$17.3 \pm 5.3$	$79.1 \pm 5.3$
Hypothalamus	$21.7 \pm 6.9$	$83.6 \pm 2.9$
Cerebellum	$23.7 \pm 4.4$	$81.9 \pm 4.4$
Pons	$14.5 \pm 3.2$	$90.6 \pm 3.6$

2). Synaptosomes from cortex, cerebellum and hypothalamus showed a peak equalling at 0.9–1.2 M sucrose. The hypothalamus also yielded a smaller secondary peak at 1.7 M sucrose. A similar distribution pattern was observed for  $^{35}\text{S}$ .

#### Release of synaptosomes

Following lysis, the synaptosomes from the cortex, hypothalamus and cerebellum lost 20% of their  $^{35}\text{S}$  content which appeared in the supernatant layer. The percentage soluble  $^{35}\text{S}$  in the pons was slightly lower about 15% (Table I).

#### Subcellular patterns of $^{35}\text{S}$ -incorporation into synaptosomes

Radioactive sulphur was found to be incorporated in several types of compounds isolated by fractionation of the crude synaptosomal pellet (Table II). The cortex, hypothalamus and cerebellum show a very similar pattern: roughly one third is represented by lipid-bound, one third by sulphomucopolysaccharide-bound and about one third was recovered in the supernatant layer after SMPs precipitation. 3–4% remained in the residual protein precipitate, probably partly due to adsorption and partly to incomplete breakdown of the SMP-protein linkage. In the synaptosomal pellet isolated from the pons the prevalent form of  $^{35}\text{S}$  was found incorporated in the lipid fraction.

#### Electrophoretic characterization of the $^{35}\text{S}$ -labelled compounds

The SMPs isolated from synaptosomes of different brain regions showed essentially the same electrophoretic pattern. On the barium acetate electrophoretogram 2 maxima of  $^{35}\text{S}$  radioactivity were detected. The Rf of one of them corresponded to the Rf of the reference compound sulphate, the other peak lay between the reference compounds of dermatan sulphate and chondroitin sulphate, thus pointing to the possible presence of a dermatan sulphate-chondroitin sulphate hybrid. The result of the separation of the SMPs on cellulose columns is represented in Fig. 3. On fractionation of the SMPs on cellulose columns

Fig. 2. Distribution of radioactivity in synaptosomes (labelled *in vivo* with H-NA) from a) cerebral cortex, b) hypothalamus and c) cerebellum in continuous sucrose density gradients ranging from 0.3–1.6 M sucrose. The gradients were centrifuged at 75 000  $\times$  g for 120 min. Animals were injected 24 h previously with  $^{35}\text{S}$ -MCP. (—) depicts tritium distribution and (---)  $^{35}\text{S}$  distribution. Representative diagrams have been chosen from 3–5 experiments.

TABLE II Percentage distribution of radioactive sulphur among different groups of compounds from synaptosomes of different brain regions. Precipitate—residual protein precipitate after papain digestion. Supernatant—supernatant layer after SMPS precipitation. The values are mean  $\pm$  S.E. of 3–4 expts.

Brain region	Radioactive sulphur $\mu\text{g } \%$			
	Lipid-bound	Non-lipid bound		
		Precipitate	Supernatant	SMPS
Cortex	26.2 $\pm$ 1.7	4.3 $\pm$ 0.4	34.5 $\pm$ 0.7	34.8 $\pm$ 1.3
Hypothalamus	41.6 $\pm$ 4.9	3.1 $\pm$ 0.1	22.9 $\pm$ 2.7	32.3 $\pm$ 2.2
Cerebellum	38.4 $\pm$ 2.4	4.1 $\pm$ 0.2	26.6 $\pm$ 1.2	30.9 $\pm$ 1.2
Pons	38.4 $\pm$ 2.1	2.8 $\pm$ 0.02	16.9 $\pm$ 0.5	21.8 $\pm$ 1.9

according to Svejcar and van Robertson where the different SMPSs are eluted from a column in a certain order one maximum of  $^{35}\text{S}$  radioactivity occurred in the fourth fraction corresponding to chondroitin-4-sulphate, resp. chondroitin-6-sulphate another peak in the third fraction corresponding to heparan sulphate (Fig. 3). Similar results from all four brain regions showed essentially the same fractionation pattern (Fig. 1). A difference was demonstrated by fractionating SMPSs isolated from lysed and non-lysed synaptosomal pellets. The high amounts of  $^{35}\text{S}$ -labelled material sometimes appearing in the first fraction indicated in all probability breakdown products. When the SMPS precipitate was treated before fractionation with chondroitinase AC the amount of  $^{35}\text{S}$ -labelled compounds in the fourth and fifth fractions significantly diminished while it was correspondingly increased in the first fraction. For precise characterization of the chondroitin sulphate it would be necessary to use treatment with different chondroitinases because the elution pattern of chondroitin sulphates can be slightly shifted depending on the molecular weight and degree of hybridization. Since, because of lack of material only chondroitinase AC, slightly contaminated with chondroitinase B, was used, it was not possible to distinguish with certainty between chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate.

Nevertheless, based on both identification procedures, the results point to chondroitin sulphate, resp. a hybrid chondroitin-dermatan sulphate and to heparan sulphate as the main SMPSs occurring in the synaptosome pellet from all 4 brain regions.

The  $^{35}\text{S}$ -labelled lipids of the chloroform-methanol extract were shown on thin layer chromatography to correspond to reference samples of sulphatides.

An attempt was also made to characterize the group of  $^{35}\text{S}$ -labelled compounds which appeared in the supernatant layer after SMPS precipitation. The elution pattern of this fraction on a Sephadex G 50 column was compared to the elution patterns of glycoproteins and mucopolysaccharides published by Margolis and Margolis (1970). It seems likely that the  $^{35}\text{S}$ -labelled compounds recovered in this fraction correspond to sulphated glycoproteins.

### Discussion

The significant decrease in the uptake of  $^3\text{H}$  NA in synaptosomal fractions of cortex and hypothalamus from animals pretreated at birth with 6-OH DA has been interpreted as a

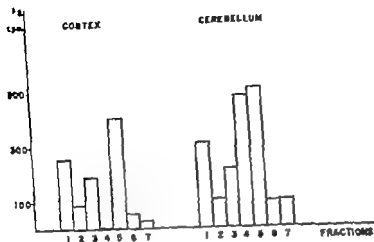


Fig. 3

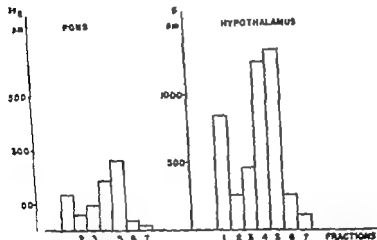


Fig. 3b.

Fig. 3 Fractionation of  $^3\text{H}$ -labelled SMPS from different regions of rat brain on cellulose columns eluted with solvents of rising ionic strength. Order of elution: F - keratan sulphate or breakdown products of sulphated polysaccharide SMPS; F - hyaluronic acid; F - heparan sulphate; F - chondroitin-4-sulphate; F - chondroitin-6-sulphate; F - dermatan sulphate; F - heparin. Representative diagrams have been chosen from 3-5 experiments.

proliferation and selective destruction of noradrenergic nerve terminals in these regions of the brain (Sachs and Jonsson 1972, Singh and de Champlain 1972). A reasonable suggestion to this phenomenon is that the dorsal ascending NA bundle, originating from the locus coeruleus cell group in the pons and supplying nerve terminals to the cerebral cortex, is preferentially attacked by 6-OH DA treatment (Jonsson *et al.* 1974). Associated with this effect is also dramatic increase in  $^3\text{H}$ -NA uptake in the pons-medulla region. It has been suggested that an increased number of nerve terminals, due to an increased outgrowth from

TABLE II Percentage distribution of radioactive sulphur among different groups of compounds eluted from synaptosomes of different brain regions. Precipitate = residual protein precipitate after papain digestion. Supernatant = supernatant layer after SMPS precipitation. The results are mean  $\pm$  S.E. of 3-4 expts.

Brain region	Radioactive sulphur $^{35}\text{S}$ %			
	Lipid-bound	Non-lipid bound		
		Precipitate	Supernatant	SMPS
Cortex	46.2 $\pm$ 1.7	4.3 $\pm$ 0.4	34.5 $\pm$ 0.7	34.8 $\pm$ 1.3
Hypothalamus	41.6 $\pm$ 4.9	3.1 $\pm$ 0.1	22.9 $\pm$ 2.7	32.3 $\pm$ 2.2
Cerebellum	38.4 $\pm$ 2.4	4.1 $\pm$ 0.2	26.6 $\pm$ 1.2	30.9 $\pm$ 1.3
Pons	38.4 $\pm$ 2.1	2.8 $\pm$ 0.02	16.9 $\pm$ 0.5	21.8 $\pm$ 1.9

according to Svejcar and van Robertson where the different SMPSs are eluted from column in a certain order one maximum of  $^{35}\text{S}$  radioactivity occurred in the fourth/fifth fraction, corresponding to chondroitin-4-sulphate, resp. chondroitin-6-sulphate, another peak in the third fraction corresponding to heparan sulphate (Fig. 3). SM from all four brain regions showed essentially the same fractionation pattern (Fig. 3). A difference was demonstrated by fractionating SMPSs isolated from lysed and non-lysed synaptosomal pellets. The high amounts of  $^{35}\text{S}$ -labelled material sometimes appeared in the first fraction indicated in all probability breakdown products. When the SMPS precipitate was treated before fractionation with chondroitinase AC the amount of  $^{35}\text{S}$ -labelled compounds in the fourth and fifth fractions significantly diminished while it was correspondingly increased in the first fraction. For precise characterization of the chondroitin sulphate it would be necessary to use treatment with different chondroitinases because elution pattern of chondroitin sulphates can be slightly shifted depending on the molecular weight and degree of hybridization. Since, because of lack of material, only chondroitinase AC, slightly contaminated with chondroitinase B, was used it was not possible to distinguish with certainty between chondroitin-4-sulphate, chondroitin-6-sulphate and dermatan sulphate.

Nevertheless, based on both identification procedures, the results point to chondroitin sulphate, resp. a hybrid chondroitin-dermatansulphate and to heparan sulphate as the main SMPSs occurring in the synaptosome pellet from all 4 brain regions.

The  $^{35}\text{S}$ -labelled lipids of the chloroform-methanol extract were shown on thin layer chromatography to correspond to reference samples of sulphatides.

An attempt was also made to characterize the group of  $^{35}\text{S}$ -labelled compounds which appeared in the supernatant layer after SMPS precipitation. The elution pattern of this fraction on a Sephadex G-50 column was compared to the elution patterns of glycoproteins and mucopolysaccharides published by Margolis and Margolis (1970). It seems likely that the  $^{35}\text{S}$ -labelled compounds recovered in this fraction correspond to sulphated glycoproteins.

### Discussion

The significant decrease in the uptake of  $^3\text{H}$  NA in synaptosomal fractions of cortex and hypothalamus from animals pretreated at birth with 6-OH DA has been interpreted

the whole brain (Margolis and Margolis 1970, for references see Balasubramanian and Bacchewal 1970).

In all four regions the SMPs have been identified as chondroitin sulphate and heparan sulphate, in agreement with the results of Bacchewal *et al.* (1972) who identified these compounds in a synaptosomal preparation from the whole brain of the rat. The confirmation of identical SMPs in synaptosomes from different regions seems to indicate a common nature in the composition of brain nerve endings, the SMPs occur probably in a protonated form, as has been generally established for tissue SMPs (for references see Bruns and Rapoport 1964, Rapoport 1969, Rodén 1967). In addition, Vos, Kuriyama and Roberts (1969) find the SMPs mainly located in synaptosomal vesicles isolated from whole brain preparations.

The amounts of  $^{35}\text{S}$ -containing material obtained from the different brain regions were sufficient for quantitative determination of the SMPs or a closer characterization of their cation moieties probably present. We refrain therefore from any discussion about their amine binding abilities. We refer in this respect to two papers presently under preparation concerning the amine storing capacity *in vitro* of a protamine-heparin complex and of an ATP-free material from bovine medullary granules (Uvnäs and Åborg 1975).

Financial support from Swedish Medical Research Council (project No. 875-04X 39-11A) and from K.L. is gratefully acknowledged.

## References

- ÅBERG, C. H., O. FELLÖN, R. NORRÄ and B. UVNÄS. A sulphomucopolysaccharide-protein complex in the adrenergic vesicle fraction from nerves and tissues. *Acta physiol. scand.* 1972, 86, 427-429.
- ÅBERG, C. H., K. A. BALASUBRAMANIAN, A. S. BALASUBRAMANIAN, M. SOGIN, E. GILSON and E. V. CHANDRASEKHARAN. Chemistry and metabolism of glycomucopolysaccharides of the nervous system. *Advanc. exp. Med. Biol.* 1972, 25, 51-71.
- BALASUBRAMANIAN, A. S. and B. K. BACCHAW. Soluble metabolism in brain. *Annu. Rev.* 1970, 20, 341-368.
- BACCHAW, J. S. and J. M. WEISS. *Mucopolysaccharides*. Elsevier Publ. Comp. 1964.
- DE PRADA, M. K., von BELLERICH and A. FLEISCHER. Storage of biogenic amines in blood platelets and adrenal medulla. *Hemato-Schmidberg's Arch. Pharmacol.* 1972, 273, 315-322.
- FELLÖN, O., R. NORRÄ and B. UVNÄS. The presence of mucopolysaccharide-protein complex in adrenal medullary cell granules. *Acta physiol. scand.* 1971, 83, 286-288.
- GILSON, O., CH. PICOT, K. FLOIS and CH. BAGES. Changes in the development of central noradrenergic neurons following neonatal administration of 6-hydroxydopamine. *J. Neurochem.* 1974, 22, 419-424.
- ISHIDA, K. and T. A. OKADA. Incorporation of  $^{35}\text{S}$ -sulphate into developing mouse brain. subcellular fractionation and electron microscopical studies. *Exp. Neurol.* 1971, 30, 18-29.
- MARGOLIS, R. K. and R. U. MARGOLIS. Sulphated glycopeptides from rat brain glycoproteins. *Biochemistry* 1970, 9, 4389-4396.
- MARGOLIS, R. U. and R. K. MARGOLIS. Isolation of chondroitin sulphate and glycopeptides from chondroitin granules of adrenal medulla. *Biochem. Pharmacol.* 1973, 22, 2185-2197.
- MARGOLIS, R. K., S. O. JAMES and R. U. MARGOLIS. Stimulation by acetylcholine of sulphated mucopolysaccharide release from the perfused rat adrenal gland. *Mol. Pharmacol.* 1973, 9, 590-594.
- OLSON, I. S., GARDILL and S. THUNELL. Biosynthesis of glycomucopolysaccharides in human leucocytes. *Biochem. Biophys. Acta (Amst.)* 1968, 165, 308-323.
- PICOT, C. and G. JONARD. Temperature-dependent changes of equilibrium characteristics of catecholamine synaptosomes in various density gradients. *Mol. Biol.* 1974, 32, 260-268.
- RAPOPORT, S. M. *Molecular Biology of the Nervous System*. VEB Verlag Volk und Gesundheit, Berlin 1968, p. 684-696.
- RODÉN, L. Structure of mucopolysaccharide-protein complexes. *Abstracts from The Seventh Int. Congress of Biochemistry*, Tokyo 1967 Vol. 1, 69-70.

the locus coeruleus would possibly at least partly explain this observation (Jonsson *et al.* 1974).

Coupled with the decreased  $^3\text{H}$  NA uptake in cortical and hypothalamic synaptosomes there was also a decreased  $^{35}\text{S}$  content (down to 65–70% of control of these subcellular particles). Such concurrent decreases of both the tritium and sulphur label indicate that part of the  $^{35}\text{S}$  component is localized to adrenergic vesicles and might be involved in the binding and storage of catecholamines within synaptosomes. In the pons-medulla region, however the increased  $^3\text{H}$  NA uptake was not associated with increased  $^{35}\text{S}$ -content: no change between control and denervated animals was observed. Since this region of the brain has a high myelin content and about 60% of the incorporated sulphur was found associated with the lipid fraction of the synaptosomes (Table II), a change in  $^{35}\text{S}$  labelled SMPS might have escaped attention as total  $^{35}\text{S}$  was measured. Also the possibility should be kept in mind that the absence of increase of SMPS might partly be related to the formation of axon terminals with relatively few and/or immature granules with a low content of SMPS. Observations from the density gradient experiments showed that much of the sulphur corresponded very closely to the main synaptosome distribution as depicted by the tritium curve. The pons was not used in this part of the study because the high lipid content would too strongly interfere with the  $^{35}\text{S}$ -labelled SMPS distribution. Of the three regions examined on density gradients the hypothalamus showed a heavier type of synaptosomes equilibrating at 1.20 M sucrose, whilst those of the cerebral cortex and cerebellum were less dense as equilibrated at about 1.10 M sucrose (Pycock and Jonsson 1974). A smaller additional peak of radioactive sulphur concomitant with tritium was observed in the distribution pattern of the hypothalamus. This secondary peak could possibly represent small catecholamine storage vesicles (equilibrating at around 0.75–0.60 M sucrose) released during the preparation and resuspension of the synaptosomes before density gradient centrifugation. Indeed  $^3\text{H}$  NA labelled synaptosomes subjected to mild lysis procedure in hypotonic sucrose before gradient centrifugation produce a broad storage vesicle peak in the range 0.75–0.55 M sucrose (Pycock, unpublished observations).

The 20% loss of radioactive sulphur following complete lysis of the crude synaptosome fraction with distilled water indicates the presence of a soluble releasable component of sulphur-containing compounds as shown for the acetylcholine stimulated perfused adrenal gland of the cat (Margolis, Jaanus and Margolis 1973). The possible association of this  $^{35}\text{S}$ -containing material with catecholamine storage remains to be shown. In addition, there may well be a small fraction of insoluble SMPS associated with the membrane fragments in suspension. Experiments with  $^3\text{H}$  NA labelled synaptosomes show that 80–90% of radioactive activity is releasable by lysis in distilled water.

The observed parallel decrease of  $^3\text{H}$  NA uptake into synaptosomes and their  $^{35}\text{S}$  content after 6-OH DA treatment and the concomitant distribution of  $^3\text{H}$  and  $^{35}\text{S}$  on gradient prompted the question about the chemical identity of the sulphur labelled compounds, resp. compounds. Indeed, several groups of  $^{35}\text{S}$  labelled compounds were verified in different synaptosome fractions from the cortex, hypothalamus and cerebellum: the  $^{35}\text{S}$  label was almost evenly distributed between sulphatides, sulphated glycoproteins and SMPSs. The three groups of compounds have been in recent years repeatedly identified in preparation



## The Interstitial Space of Adipose Tissue as Determined by Single Injection and Equilibration Techniques

By

BIRGITTA LINDE and GUY CHENOLAS

Received 18 April 1975

### Abstract

LINDE, B. and G. CHENOLAS. The interstitial space of adipose tissue as determined by single injection and equilibration techniques. *Acta physiol. scand.* 1975. 95. 383-390.

The interstitial  $^{14}\text{C}$ -sucrose space was determined in the subcutaneous adipose tissue of the dog using both single injection-indicator dilution technique and an equilibration-tissue sampling method.  $^{125}\text{I}$ -albumin and  $\text{Cr}$ -labelled erythrocytes served as intravascular indicators. The conventional extrapolation method for single injection curves yielded space values several times lower than those obtained by the equilibration technique, although sampling was continued until the efferent outflow samples contained less than 1% of peak  $^{14}\text{C}$ -sucrose activity. This discrepancy may be due to long transit times, for which the conventional extrapolation procedure does not account. An extrapolation procedure is proposed based on the assumption that the area under the normalized extra- and intravascular curves should be equal. By this procedure as well as by the equilibration technique the interstitial  $^{14}\text{C}$ -sucrose space in adipose tissue measures approximately 18 ml/100 g<sup>-1</sup>. The single injection technique does not appear applicable for space determination in adipose tissue during sympathetic nerve stimulation (3-7 Hz), possibly due to "trapping" of the tracer within the tissue.

The single injection-indicator dilution technique (Meier and Zierler 1954) was used to measure the size of the interstitial space in the subcutaneous adipose tissue of the dog. Earlier studies (Öberg and Rosell 1967) have suggested that decrease in the size of this compartment may occur during sympathetic nerve stimulation. However the application of the single injection technique to determine space in adipose tissue involved methodological problems. Therefore, a somewhat more detailed study of this method as a measure of extra-vascular space in adipose tissue was carried out. Spaces obtained by the single injection technique were compared to those measured by an equilibration-tissue sampling method.

### Methods

13 female mongrel dogs, fasted overnight, were anesthetized with sodium pentobarbital, 30 mg/kg i.v. supplemented as necessary. All animals were tracheotomized and artificial positive pressure breathing was

Present address: Dept of Chemical Engineering, M.I.T. Cambridge, Mass. 02139, U.S.A.

- SACHS, CH. and G. JONSSON, Degeneration of central noradrenaline neurons after 6-hydroxy newborn animals. *Res. Commun. Chem. Path. Pharmac.* 1972. 1 203-220.
- SINGH, B. and J. DE CHAMPLAIN, Altered ontogenesis of central noradrenergic neurons follows treatment with 6-hydroxydopamine. *Brain Res.* 1972. 48. 432-437.
- SVEDCAR, J. and W. VAN ROBERTSON, Micro separation and determination of mammalian ac aminoglycans (mucopolysaccharides). *Analyt. Biochem.* 1967. 18. 333-350.
- UVNÄS, B., An attempt to explain nervous transmitter release as due to nerve impulse-induced change. *Acta physiol. scand.* 1973. 87. 168-173.
- VOX, J. K. KURIYAMA and E. ROBERTS, Distribution of acid mucopolysaccharides in subcellular of mouse brain. *Brain Res.* 1969. 12. 172-179.
- WEISLER, E., Analytical and preparative separation of acidic glycosaminoglycans by cation barium acetate. *Analyt. Biochem.* 1968. 26. 439-444.
- WHITTAKER, U. P. The synaptosome. In *Handbook of Neurochemistry* Vol. 2. Ed. A. Lajtha. Press, New York 1969.

## The Interstitial Space of Adipose Tissue as Determined by Single Injection and Equilibration Techniques

By

BIRGITTA LINDE and GUY CHENOLAT

Received 18 April 1975

### Abstract

LINDE, B. and CHENOLAT, G. *The interstitial space of adipose tissue as determined by single injection and equilibration techniques.* Acta physiol. scand. 1975 95 383-390.

Interstitial  $^{14}\text{C}$ -sucrose space as determined in the subcutaneous adipose tissue of the dog using both the single-injection dilution technique and an equilibration-tissue sampling method.  $^{251}\text{I}$ -albumin and  $^{51}\text{Cr}$ -labelled erythrocytes served as intravascular indicators. The conventional extrapolation method for the space curves yielded space values several times lower than those measured by the equilibration technique, although sampling was continued until the venous outflow samples contained less than 1% of injected  $^{14}\text{C}$ -sucrose activity. This discrepancy may be due to long transit times, for which the conventional extrapolation procedure does not account. An extrapolation procedure is proposed based on the assumption that the area under the measured extra- and intravascular curves should be equal. By this procedure as well as by the equilibration technique the interstitial  $^{14}\text{C}$ -sucrose space in adipose tissue measures approximately 10 ml  $100\text{ g}^{-1}$ . The single injection technique does not appear applicable for space determinations in adipose tissue during sympathetic nerve stimulation (3-7 Hz), possibly due to "trapping" of the tracer in the tissue.

The single injection-indicator dilution technique (Meier and Zierler 1954) was used to measure the size of the interstitial space in the subcutaneous adipose tissue of the dog. Earlier studies (Öberg and Rosell 1967) have suggested that a decrease in the size of this compartment may occur during sympathetic nerve stimulation. However, the application of the single injection technique to determine space in adipose tissue involved methodological problems. Therefore, a somewhat more detailed study of this method as a measure of extravascular space in adipose tissue was carried out. Spaces obtained by the single injection technique were compared to those measured by an equilibration-tissue sampling method.

### Methods

Is female mongrel dogs, fasted overnight, were anaesthetized with sodium pentobarbital, 30 mg/kg supplemented as necessary. All animals were tracheotomized and artificial positive pressure breathing was

given when necessary to maintain normal blood gas values. A subcutaneous adipose tissue preparation of the inguinal region according to Rosell (1966) was used. The tissue was totally freed from para-tissues, including the skin, except for an artery and vein. The mixed nerve to the tissue was sectioned. Flow was measured using a silicone-filled drop counter inserted into the artery. Heparin in doses of 2.5 kg was used as an anticoagulant. Blood flow and blood pressure were recorded continuously on a polygraph. The temperature of the preparation was kept at 37–38°C with the aid of heating lamp tissue was kept moist by covering with a plastic sheet and saline soaked gauze pads.

A *equilibrium-tissue sampling* procedure was used to determine the sucrose space in ax expts and Phelps (1966). The tissue was artificially perfused from a reservoir according to the technique of Rosell (1962). 100–250  $\mu$  Ci  $^{14}$ C-sucrose (The Radiochemical Centre, Amersham) and approx. 50  $\mu$  Ci  $^{51}$ Cr labelled red cells were mixed with about 100–300 ml of the dog's blood in the perfusion tank which was maintained at 38°C. Thus, an *in situ* perfusion was established with the effluent coming being discarded. Labelling of the red blood cells was performed according to Owen (1959) using  $^{51}$ CrO (Atomenergik, Studia 14). The tissue was perfused for approximately 90 min (range 50–150 min) at an average blood flow of 6.6 ml min<sup>-1</sup> 100 g<sup>-1</sup> and at an average pressure of 140 mm Hg at the inflow site. Arterial and venous samples were collected every 10 to 15 min for hematocrit determinations and liquid scintillation counting. No statistically significant difference was found in the  $^{51}$ Cr between arterial and venous blood after 90 min of perfusion. At the end of the perfusion period the artery and vein were simultaneously clamped and cut and the tissue was immediately transferred into liquid nitrogen. From the frozen fat tissue, pieces of 1–3 g were cut after removing a thin layer from the surface. The fat pieces were dissolved in Solvène<sup>®</sup> 350 (Packard Instrument) overnight at 60°C, after which 1 ml of the liquid were prepared for liquid scintillation counting (Packard Tri Carb 3375). The samples for the experiment were run together with quenched standards. The analytical errors, determined as the coefficient of variation for a single determination, were 0.8% for  $^{14}$ C and 4.6% for  $^{51}$ Cr.

Using the assumption that sucrose had totally equilibrated with the tissue, and that the sucrose concentration in the same in plasma and in interstitial fluid, the total sucrose space (properly expressed in ml equivalent plasma 100 g<sup>-1</sup> but for simplicity written as ml 100 g<sup>-1</sup>) was calculated as follows:

$$\text{Total sucrose space (ml 100 g}^{-1}\text{)} = \frac{\text{cpm } ^{14}\text{C}}{\text{cpm } ^{14}\text{C}} \frac{100 \text{ g tissue}}{\text{ml plasma}}$$

Calculation of plasma volume was performed according to the following:

$$\text{Plasma volume (ml 100 g}^{-1}\text{)} = \frac{\text{cpm } ^{51}\text{Cr}}{\text{cpm } ^{51}\text{Cr}} \frac{100 \text{ g tissue} \times (1 \text{ Hct})}{\text{ml blood} \times \text{ml blood}}$$

assuming the venous hematocrit to be equal to the tissue hematocrit. Interstitial sucrose space = sucrose space – plasma volume.

The *single injection-indicator dilution technique* (Meier and Zierler 1954) using  $^{125}$ I-albumin as the vascular indicator was used in ax expts. performed during autoperfusion of the tissue.  $^{125}$ I-albumin (1.5  $\mu$  Ci of each approximately 75  $\mu$ l) were injected over one s through rubber tubing inserted into the arterial inflow cannula. 30 to 50 venous samples were collected dropwise into scintillation vials 3.5 to 35 min after the injection.

In order to avoid recirculation of radioactive blood during the sampling period, the cross circulation was not returned to the animal until after the end of each sampling period. In high blood flow expts. the blood was taken from the animal before the injection and returned during the sampling period to keep the pressure constant. This was less of a problem since relatively large dogs were used (mean b.w. 17 kg). To make the sampling times shorter prostaglandin E<sub>2</sub> was administered in 2 expts. to increase the flow rate.

Stimulation of the sympathetic nerve supply (12 V 2 ms, 3–7 Hz) was performed via bipolar electrodes with square wave impulses from a Grass stimulator. Injections were performed approximately 1 min after the beginning of stimulation.

Assuming that mixing of blood and indicator was complete in the arterial cannula, total sucrose and plasma volume were calculated from the curves describing venous concentration versus time, according to  $V = Q_p \bar{t}$  (Meier and Zierler 1954), (where V is the volume of the system in ml equivalent plasma 100 g<sup>-1</sup>,  $Q_p$  is the plasma flow rate ml min<sup>-1</sup> 100 g<sup>-1</sup> and  $\bar{t}$  the mean transit time in min for the indicator through the system). The interstitial sucrose space was calculated as the difference between total sucrose space and the plasma volume (PV). Corrections were made for the volume of the sampling cannula which measured approximately 0.5 ml (Zierler 1962).

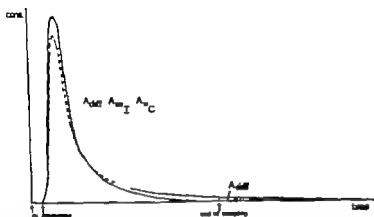


Fig. 1. Schematic picture of venous concentration-time curves for  $^{125}\text{I}$ -albumin (the intravascular indicator  $\rightarrow$ ) and  $^{14}\text{C}$ -sucrose (the extravascularly diffusing tracer,  $\cdots$ ) following single arterial injection. The two concentration curves have been normalized with respect to those of the fixed injected. The "tail" of the  $^{14}\text{C}$  curve is obtained by monoexponentially extrapolating ( $\cdots$ ) to the base line. The area under the extrapolated part of the curve ( $A_{\text{diff}}$ ) equals the difference in area between the totally-recovered  $^{125}\text{I}$  curve ( $A_{125\text{I}}$ ) and the recovered part of the  $^{14}\text{C}$  curve ( $A_{14\text{C}}$ ).

In the single injection experiments sampling was performed as each study until the  $^{125}\text{I}$  activity could no longer be distinguished from the background level. The  $^{14}\text{C}$  activity however was somewhat (0.09–0.65% the peak activity) above the background level at the end of the sampling period. Therefore extrapolation of the  $^{14}\text{C}$  curve had to be used in order to obtain the mean transit time. Extrapolation was performed in two ways, firstly by the conventional method of Hamilton *et al.* (1932), using the time intervals just before and after the sampling as basis for the extrapolation. Secondly extrapolation was performed according to method shown in Fig. 1 which is based on the assumption that  $^{14}\text{C}$  should recover in the venous out-

TABLE 1. Total and interstitial  $^{14}\text{C}$ -sucrose space determined by an equilibration technique in dog subcutaneous adipose tissue. Blood flow, mean arterial blood pressure, venous hematocrit, sucrose space and plasma volume are given, the two latter as means  $\pm$  S.E. for each expt. together with mean  $\pm$  S.E. for the whole material.

Expt.	Blood flow ml/min/100 g $^{-1}$	Mean arterial blood pressure mm Hg	Venous Hct	Plasma volume ml/100 g $^{-1}$	Total sucrose space ml/100 g $^{-1}$	Interstitial sucrose space ml/100 g $^{-1}$
2	3.4	175	32	$8.2 \pm 0.7$	$17.8 \pm 1.8$	$9.6 \pm 1.2$
7	9.9	120	47	$5.0 \pm 1.2$	$12.1 \pm 1.7$	$7.1 \pm 1.0$
2	3.8	100	28	$9.7 \pm 0.1$	$20.0 \pm 2.0$	$10.3 \pm 2.0$
5	4.0	130	37	$4.2 \pm 0.2$	—	—
6	8.1	155	40.5	$4.4 \pm 1.2$	$15.1 \pm 2.3$	$10.7 \pm 1.5$
7	1.2	175	31	—	$23.5 \pm 2.1$	—
5	8.9	170	49.5	$5.2 \pm 0.8$	$4.0 \pm 2.7$	$18.8 \pm 2.2$
11	19.0	100	40	$7.9 \pm 1.9$	$18.2 \pm 2.1$	$10.3 \pm 1.2$
4	7.9	140	48	$8.3 \pm 3.5$	$15.7 \pm 1.9$	$7.4 \pm 1.8$
Whole material				$6.6 \pm 0.8$ (8)	$18.3 \pm 1.5$ (-8)	$10.6 \pm 1.5$ (n 7)

Equilibration not complete.  
Too little  $^{14}\text{C}$  in the blood.

TABLE II. Total and interstitial  $^{14}\text{C}$  sucrose space determined by the single injection technique in dog sartorius. In Fig. 1 time of sampling, time used for start of extrapolation, venous hematocrit, plasma  $\text{Cr}$ , extravascular tracer are given for each individual injection as well as mean  $\pm$  S.E. in the last

Exp	Inj	Time of sampling min	Time used for start of extrapol. min	Venous Hct	Plasma flow ml $\text{min}^{-1}$ $100 \text{ g}^{-1}$	Plasma volume ml $100 \text{ g}^{-1}$
1	1	5.5	5.5	46	9.3	48
	2	5.5	5.5	45	9.1	44
	3	5.5	5.5	45.5	8.9	41
2	1	5.5	5.5	8.5	17.6	71
	2	5.5	5.5	28	16.8	85
3	1	5.5	5.5	40	17.5	71
	2	5.5	5.5	39	17.7	73
4	1	5.5	5.5	48	8.9	54
	2	5.5	5.5	44	9.0	51
5	1	20	15	42	5.4	64
			20			
			13	41	4.8	62
6	1	35	10	43	6.1	77
			35			
			18	42	5.8	71
	2	35	35			
Whole material $\bar{x}$					10.5	62
(n = 6)					S.E.	0.9

Value at end of  $^{125}\text{I}$  curve.

flow to the same extent as  $^{125}\text{I}$ . Thus, the area of the  $^{125}\text{I}$  curve is taken to represent 100% recovery of extravascular indicator. Careful registration of the background activity as well as of the concentration of the fluid injected is essential in this connection. Several blanks were taken between injections. The concentrations in the injected fluid were determined as an average of 10 samples. The error in the determination of the ratio between  $^{14}\text{C}$  and  $^{125}\text{I}$  in the injected fluid expressed as the coefficient of variation for single determination was 1.7%.

The  $^{14}\text{C}$  curve was monoexponentially extrapolated from the first sample containing no trace of  $^{14}\text{C}$  or from a later sample, in such a way that the area under the extrapolated part of the curve equals the difference ( $A_{\text{ext}}$ ) between the totally-recovered  $^{125}\text{I}$  curve ( $A_{^{125}\text{I}}$ ) and the only partially-recovered  $^{14}\text{C}$  curve ( $A_{^{14}\text{C}}$ ). In this way it is possible to define the whole  $^{14}\text{C}$  curve and, thus, its point of gravity can be determined, giving the  $\bar{x}$  for  $^{14}\text{C}$ -sucrose.

Standard statistical procedures were used, the paired *t*-test being employed when applicable. Data are presented as means  $\pm$  S.E.

## Results

### Equilibration technique (Table I)

After approximately 90 min of perfusion the total sucrose space measured  $19.3 \pm 1.5$  ml  $100 \text{ g}^{-1}$  tissue and its extravascular portion  $10.6 \pm 1.5$  ml  $100 \text{ g}^{-1}$ . Although the venous hematocrit was somewhat low in three of these expts., no relationship appeared in our data between space values and hematocrit. The plasma volume calculated from the tissue  $\text{Cr}$  blood content of  $\text{Cr}$ -labelled red cells measured  $6.6 \pm 0.8$  ml  $100 \text{ g}^{-1}$ . This is likely to be minimum value, since the tissue hematocrit is probably lower than the venous hematocrit.

Interst. space calculated by extrapolation according to Hamilton *et al.* (1932) and by method proposed in this paper as well as  $^{14}\text{C}$ -sucrose activity in last sample and  $A_{\text{extr}}$ . The difference in area between the intra- and interstitial spaces for each expt.

ml space ml $\text{g}^{-1}$	$^{14}\text{C}$ activity % of max	$A_{\text{extr}}$ of $A_{\text{intra}}$	Total sucrose space (Fig. 1) ml $100 \text{ g}^{-1}$	Interstitial sucrose space (Fig. 1) ml $100 \text{ g}^{-1}$
0.25	3.1		8.1	4.1
0.24	3.1		9.0	4.6
0.23	3.4		8.7	4.6
0.28	3.0		14.8	7.0
0.22	4.6		19.1	10.6
0.22	6.4		26.4	18.6
0.30	3.0		22.4	14.5
0.17	4.6		10.0	6.6
0.19	3.7		11.5	8.3
0.22	6.7		18.0	12.0
0.08	4.4		18.5	12.5
0.30	7.1		20.9	14.7
0.11	4.6		20.4	14.2
0.61	3.7		22.6	14.9
0.09	1.4		23.3	15.6
0.63	4.8		21.2	13.4
0.34	1.4		20.8	13.0
0.30 <sup>a</sup>	3.0 <sup>a</sup>		17.1	10.8
0.07	0.5		2.5	1.9

single injection technique (Table II)

plasma volume was  $6.2 \pm 0.9 \text{ ml } 100 \text{ g}^{-1}$  which is in good agreement with the value obtained from the content of  $^{51}\text{Cr}$  labelled red cells in the tissue. The coefficient of variation for a single determination of the plasma volume was 3.4%.

Interstitial space extrapolation according to Hamilton *et al.* (1932). After 5–13 min of observation, which was enough for complete washout of the intravascular indicator the  $^{14}\text{C}$ -sucrose activity in the venous outflow was only about 0.3% of the peak activity. Despite this low concentration of the extravascular tracer the interstitial space, calculated on the basis of Hamilton's method for extrapolation, measured only  $2.5 \text{ ml } 100 \text{ g}^{-1}$ . This is several times smaller than the corresponding value obtained by the equilibration technique. When the time of observation was extended (expts. 5 and 6), larger space values were obtained by an extrapolation method.

Interstitial space, extrapolation according to Fig. 1. Using this method of extrapolation, from the first sample where the  $^{125}\text{I}$ -albumin activity was zero, the interstitial sucrose space measured  $10.8 \pm 1.9 \text{ ml } 100 \text{ g}^{-1}$  and the total sucrose space  $17.1 \pm 2.5 \text{ ml } 100 \text{ g}^{-1}$ . These values are similar to those obtained by the equilibration technique. For the 4 injections in expts. 5 and 6, extrapolation was also carried out from a later point of observation. Only minor differences in the space values were observed when extrapolating from the first sample which showed no trace of  $^{125}\text{I}$ -albumin activity compared to later samples. The reproducibility

TABLE II Total and interstitial  $^{14}\text{C}$ -sucrose space determined by the single injection technique is shown in Fig. 1. Time of sampling, time used for start of extrapolation, venous hematocrit, plasma flow and  $^{14}\text{C}$  concentration are given for each individual injection as well as mean  $\pm$  S.E. in the last column.

Exp	Inj	Time of sampling min	Time used for start of extrapol. min	Venous Hct	Plasma flow ml $\text{min}^{-1}$ $100 \text{ g}^{-1}$	$^{14}\text{C}$ concentration $\mu\text{Ci ml}^{-1}$
1	1	5.5	5.5	46	9.3	4
	2	5.5	5.5	45	9.1	4
	3	5.5	5.5	45.5	8.9	4
2	1	5.5	5.5	28.5	17.6	7
	2	5.5	5.5	28	16.8	8
3	1	5.5	5.5	40	17.5	7
	2	5.5	5.5	39	17.7	7
4	1	5.5	5.5	48	8.9	3
	2	5.5	5.5	44	9.0	3
5	1	20	13	42	5.4	6
			20			
	2	20	13	41	4.8	6
6			20			
	1	35	10	43	6.1	7
			35			
	2	35	18	42	5.8	7
			35			
Whole material $\bar{x}$					10.5	6
(n = 6)					±3	8

Value at end of  $^{125}\text{I}$  curve.

flow to the same extent as  $^{125}\text{I}$ . Thus, the area of the  $^{14}\text{C}$  curve is taken to represent 100% recovery of extracellular indicator. Careful registration of the background activity as well as of the concentration of the fluid injected is essential in this connection. Several blanks were taken between injections. The concentrations in the injected fluid were determined as an average of 10 samples. The error in the determination of the ratio between  $^{14}\text{C}$  and  $^{125}\text{I}$  in the injected fluid expressed as the coefficient of variation single determination was 1.7%.

The  $^{14}\text{C}$  curve was monoexponentially extrapolated from the first sample containing no tracer or from a later sample, in such a way that the area under the extrapolated part of the curve equals the difference ( $A_{\text{ext}}$ ) between the totally recovered  $^{125}\text{I}$  curve ( $A_{\text{int}}$ ) and the only partially-recovered  $^{14}\text{C}$  ( $A_{\text{ext}}$ ). In this way it is possible to define the whole  $^{14}\text{C}$  curve and thus, its point of gravity can be defined, giving the  $\bar{x}$  for  $^{14}\text{C}$ -sucrose.

Standard statistical procedures were used, the paired *t* test being employed when applicable. Data presented as means  $\pm$  S.E.

## Results

### Equilibration technique (Table I)

After approximately 90 min of perfusion the total sucrose space measured  $18.3 \pm 1.5$   $100 \text{ g}^{-1}$  tissue and its extravascular portion  $10.6 \pm 1.5$   $\text{ml } 100 \text{ g}^{-1}$ . Although the venous hematocrit was somewhat low in three of these expts. no relationship appeared to exist between space values and hematocrit. The plasma volume calculated from the tissue blood content of  $^{51}\text{Cr}$ -labelled red cells measured  $6.6 \pm 0.8$   $\text{ml } 100 \text{ g}^{-1}$ . This is likely to be a minimum value, since the tissue hematocrit is probably lower than the venous hematocrit.



comparisons, based on studies in skeletal muscle, was given by Lassen and Sejrsen (1971), who stated that "monoexponential extrapolation as made before the appearance of the final venous part of the outflow curve, can cause considerable error in determination of the transit time and hence of the volumes of distribution

in certain organs and tissues, e.g. in heart and skeletal muscle, but not in liver. A venous part of the extravascular space has long transit times. When these long transits are not properly accounted for, considerable errors in the volumes of distribution may result. We suggest that the discrepancy found in adipose tissue between values of the interstitial space obtained by conventional extrapolation of single injection curves and those determined by the equilibration method is caused by such long transits for which the Hamilton method of extrapolation does not account. This explanation appeared even more likely when, after extending the time of observation of the venous outflow, the space values obtained by the conventional extrapolation method showed an increase, compared to the values calculated by extrapolation from an earlier point of registration.

Theoretically the most correct way of accounting for the long transit times is certainly a method deduced by Lassen and Sejrsen (1971), where the monoexponential tail is found by simultaneous outflow and residue detection. We propose another procedure for considering the long transits, based only on outflow detection. The method requires registration of the entire curve of the intravascular tracer, the area of which is assumed to represent 100% recovery for the extravascular indicator. This assumption appears valid if the indicators are not and if changes in blood flow do not distort the curves to a different extent. However, to obtain the most accurate measurements possible, utmost care must be taken in the determination of the areas, by frequent sampling, by careful registration of the background as well as by accurate determination of the concentrations in the fluid injected. When these precautions are taken in the present study the coefficient of variation for a single determination of the interstitial space was 11%. No marked difference in the space values was seen whether extrapolation was performed early or late after the point where the  $^{125}\text{I}$ -albumin activity was zero.

Attempts to apply the single injection technique during sympathetic nerve stimulation (1-7 Hz) resulted in unreasonably high values of the interstitial space, in some cases even values above the tissue volume. This may be due partly to the systematic change of flow taking place in adipose tissue during nerve stimulation, since attempts to correct for this flow factor resulted in lower values. However, during nerve stimulation other factors may also influence the rate of outflow of the indicators. We suggest that "trapping" which has been described for the outflow of FFA from adipose tissue (Rosell 1966), may contribute to the high values, since obvious over-estimations occurred for both intra- and extravascular space.

In summary the present study demonstrates the importance of considering the longest transit times when calculating interstitial space from single injection curves in subcutaneous adipose tissue. An extrapolation procedure is proposed, based on outflow detection of an intra- and extravascular indicator. It requires registration of the entire outflow curve for the intravascular tracer, the area of which is thought to represent 100% recovery of the extravascular indicator. Using this procedure good agreement is found between space values

for the determination of the interstitial space, expressed as the coefficient of variation single determination, was 19.1%.

#### *Sympathetic nerve stimulation*

Attempts were also made to apply the single injection technique during sympathetic stimulation (3–7 Hz). Using the extrapolation procedure according to Fig. 1 quite unrealistic values for the intra- and extravascular spaces were obtained. Interstitial space values greater than the tissue volume were calculated in some cases. Since the capacitance has been shown to decrease during nerve stimulation (Öberg and Rosell 1967) the high intravascular values measured are probably due to an error in the measuring technique. During stimulated sympathetic nerves to adipose tissue, blood flow decreases initially. After some time vasoconstriction starts to diminish, resulting in a gradual increase in blood flow. This continuous flow change may partly be responsible for the high values. Attempts to correct the flow alteration, by integrating the product of venous concentration and its corresponding flow values over time, resulted in more reasonable space values in 2 injections. However, in the other injections, no obvious explanation for the high intra- and extravascular space values could be found. Therefore no further attempts were made to apply the single injection technique during nerve stimulation conditions.

### *Discussion*

The aim of the present study was initially to measure the size of the interstitial space in subcutaneous adipose tissue and to investigate whether a decrease of this space takes place during sympathetic nerve stimulation, as indicated by earlier studies (Öberg and Rosell 1967). The single injection technique (Meier and Zierler 1954), which enables repeated measurements to be made in the same tissue, was chosen in preference to equilibration methods. However, in spite of sampling until less than one per cent of the peak  $^{14}\text{C}$ -sucrose activity remained in the venous outflow the space values obtained by this technique, using the conventional extrapolation method of Hamilton *et al.* (1932), were several times smaller than previously reported from adipose tissue of other species and locations using equilibration techniques *in vitro* (Denton *et al.* 1966, Björntorp *et al.* 1966, Engthardt *et al.* 1971). These values were also much smaller than values of adipose tissue water obtained by Fredholm & Fronek (1974). Therefore the interstitial space in this tissue was determined using an equilibration—tissue sampling method (Law and Phelps 1966). The duration of perfusion was chosen to give practically complete equilibration between tissue and blood. The space values thus obtained were considerably larger than those calculated from the single injection experiments, using the conventional extrapolation method, but in good agreement with previous estimates from other studies mentioned above.

A discrepancy between extravascular spaces obtained by the conventional extrapolation of single injection curves and those determined by equilibration methods has also been observed in heart muscle, in spite of recoveries near 100% (Ziegler and Goresky 1971). In liver the space values based on extrapolation according to Hamilton *et al.* agreed well with other estimates of the extravascular space (Goresky 1963). A likely explanation for the

## Comparison of the Effects of Physical Exercise, Cold Acclimation and Repeated Injections of Isoprenaline on Rat Muscle Enzymes

By

M. N. E. HARRI and J. VALTOLA

Received 5 May 1975

### Abstract

M. N. E. and J. VALTOLA. Comparison of the effects of physical exercise, cold acclimation and repeated injections of isoprenaline on rat muscle enzymes. *Acta physiol. scand.* 1975, 95, 391-399.

The metabolic effects on rat cardiac and skeletal muscle of strenuous program of swimming, of cold acclimation and of isoprenaline treatment (0.3 mg/kg daily for 3 two-day weeks) were compared. Exercised and cold-exposed rats gained less body weight than did controls or isoprenaline-treated rats. In all treated rats the heart and the intercostal brown adipose tissue hypertrophied. The size of the adrenals increased only in isoprenaline-treated animals. Cold-acclimation and physical training increased and isoprenaline treatment reduced or did not affect the activities of succinate dehydrogenase, malate dehydrogenase and various isozymes of cardiac muscle. In skeletal muscle all treatments resulted in increased activities of most enzymes. Of the extensive enzymes analysed, only the activity of hexokinase increased in response to the treatments used. This increase was the same in cardiac as in skeletal muscle, but it was significantly greater with isoprenaline-treatment than with training or with cold-acclimation. The activities of lactate dehydrogenase and phosphocreatinase did not differ significantly. All treatments improved cold tolerance, but only swimming exercise and cold acclimation significantly increased tolerance to exercise. It is concluded that prolonged stimulation of adrenergic  $\beta$ -receptors by catecholamines is responsible for the metabolic changes observed.

**Key words:** Gastrocnemius muscle, cardiac muscle, enzyme activity, physical exercise, cold acclimation, isoprenaline treatment, cold tolerance, exercise tolerance.

It is well known that skeletal and cardiac muscle adapt to physical exercise by increasing the number and size of mitochondria and content of mitochondrial enzymes (Holloday 1973). On the other hand, attempts to demonstrate changes in the activities of enzymes involved in glycolysis have led to contradictory results (Holloday *et al.* 1971, Gollnick and Hermanson 1973). The changes in muscle enzymes as a result of physical training are very similar to those achieved by cold-acclimation (Hannon 1963, Hamilton and Ferguson 1972). Furthermore, acclimation to cold increases the amount of time a rat can swim before exhaustion (Bashashov 1960, Dawson *et al.* 1970). A relationship between physical fitness and the ability to tolerate cold has been demonstrated in humans (Adams and Heberling 1958, Heberling and Adams 1961, Keatings 1961, Lange Andersen 1966) and in rats (Strömme and Hannon 1967). On the other hand, repeated injections of isoprenaline results in improved

measured by the single injection technique and those determined by an equilibration model. Both methods give values for the interstitial space in adipose tissue of approximately 10 ml/100 g<sup>-1</sup> or about half of the body average. Application of the single injection technique to the determination of interstitial space during sympathetic nerve stimulation has not been successful in the present study.

This study was supported by grants from the Swedish Medical Research Council (875-60X 3512-40) and by the Karolinska Institutet, Stockholm.

## References

- BÄCKSTRÖM, P. B. HOGG and A. MARTINSSON, The sucrose space of human subcutaneous adipose tissue in obesity. *Acta med. scand.* 1966, 180, 123-127.
- DENTON, R. M., R. E. YORKER and P. J. RANDALL, Measurement of concentrations of metabolites in adipose tissue and effects of insulin, alloxan diabetes and adrenaline. *Biochem. J.* 1966, 100, 407-419.
- ENGELHARDT, A., H. LIEBERMANN, Th. REUTER and K. LAMACHER, Analyse der Wasser- und Lipidgehalte des menschlichen subcutanen Fettgewebes. *Z. Anal. Chem. Berchem.* 1971, 356-360.
- FREDHOLM, B. B. and A. FRONK, High energy phosphate compounds in adipose tissue: The effect of hemorrhage. *Acta physiol. scand.* 1974, 91, 165-171.
- GORESKY, C. A., A linear method for determining liver sinusoidal and extravascular volumes. *Amer. J. Physiol.* 1963, 204, 626-640.
- HAMILTON, W. F. I. W. MOORE, I. M. KIDMAN and J. M. SPURLING, Studies on the circulation. IV. Further analysis of the injection method, and of changes in hemodynamics under physiological and pathological conditions. *Amer. J. Physiol.* 1932, 99, 534-551.
- LASSEN, N. A. and P. SEVERIN, Monoexponential extrapolation of tracer clearance curves in kinetic analysis. *Circulat. Res.* 1971, 29, 76-87.
- LAW, R. O. and C. F. PHELPS, The size of the sucrose, raffinose, and inulin spaces in the gastrocnemius muscle of the rat. *J. Physiol. (Lond.)* 1966, 186, 547-557.
- MEIER, P. and K. L. ZIERLER, On the theory of the indicator-dilution method for measurement of blood flow and volume. *J. appl. Physiol.* 1954, 6, 731-744.
- ÖBERG, B. and S. ROSELL, Sympathetic control of consecutive vascular sections in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1967, 71, 47-56.
- OWEN, C. A., *Diagnostic radiology*. Charles C. Thomas, Springfield, USA 1959, p. 163.
- RENKIN, E. M. and S. ROSELL, The influence of sympathetic adrenergic vasoconstrictor nerves on transport of diffusible solutes from blood to tissues in skeletal muscle. *Acta physiol. scand.* 1962, 54, 23-36.
- ROSELL, S., Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation. *Acta physiol. scand.* 1966, 67, 343-351.
- ZIERLER, W. H. and C. A. GORESKY, Transcapillary exchange in the working left ventricle of the rat. *Circulat. Res.* 1971, 24, 181-207.
- ZIERLER, K. L., Theory of use of indicators to measure blood flow and extracellular volume and calculation of transcapillary movement of tracer. *Circulat. Res.* 1963, 12, 464-471.
- ZIERLER, K. L., Theoretical basis of indicator-dilution methods for measuring flow and volume. *Circulat. Res.* 1962, 10, 393-407.

## Comparison of the Effects of Physical Exercise, Cold Acclimation and Repeated Injections of Isoprenaline on Rat Muscle Enzymes

By

M. N. E. HARRI and J. VALTOLA

Received 5 May 1975

### Abstract

HARRI, M. N. E. and J. VALTOLA. Comparison of the effects of physical exercise, cold acclimation and repeated injections of isoprenaline on rat muscle enzymes. *Acta physiol. scand.* 1975, 95, 391-399.

Metabolic effects on rat cardiac and skeletal muscle of a strenuous program of swimming, of cold acclimation and of isoprenaline treatment (0.3 mg/kg daily for 31 a-day weeks) were compared. Exercised and cold-exposed rats gained less body weight than did controls or isoprenaline-treated rats. In all treated rats the heart and the interscapular brown adipose tissue hypertrophied. The size of the adrenals increased only in isoprenaline-treated animals. Cold-acclimation and physical training increased and isoprenaline treatment reduced or did not effect the activities of succinate dehydrogenase, malate dehydrogenase and cytochrome oxidase of cardiac muscle. In skeletal muscle all treatments resulted in increased activities of the same enzymes. Of the aerobic enzymes analyzed, only the activity of hexokinase increased in response to the treatments used. This increase was the same in cardiac as in skeletal muscle, but it was significantly greater with isoprenaline-treatment than with training or with cold-acclimation. The activities of lactate dehydrogenase and phosphofructokinase did not differ significantly. All treatments improved cold resistance, but only swimming exercise and cold acclimation significantly increased tolerance to exercise. It is concluded that prolonged stimulation of adrenergic  $\beta$ -receptors by catecholamines is responsible for the metabolic changes observed.

**Key words:** Gastrocnemius muscle, cardiac muscle, enzyme activity, physical exercise, cold acclimation, swimming, exercise, cold tolerance, exercise tolerance.

It is well known that skeletal and cardiac muscle adapt to physical exercise by increasing the number and size of mitochondria and content of mitochondrial enzymes (Hollotry 1973). On the other hand, attempts to demonstrate changes in the activities of enzymes involved in glycolysis have led to contradictory results (Hollotry *et al.* 1971, Gollnick and Harrison 1973). The changes in muscle enzymes as a result of physical training are very similar to those achieved by cold-acclimation (Harrison 1963, Hamilton and Ferguson 1972). Furthermore, acclimation to cold increases the amount of time a rat can swim before exhaustion (Bashbeshova 1960, Dawson *et al.* 1970). A relationship between physical fitness and the ability to tolerate cold has been demonstrated in humans (Adams and Heberling 1954, Heberling and Adams 1961, Kestingo 1961, Lange Andersen 1966) and in rats (Stratton and Hammond 1967). On the other hand, repeated injections of isoprenaline results in improved

measured by the single injection technique and those determined by an equilibration method. Both methods give values for the interstitial space in adipose tissue of approximately 10 ml 100 g<sup>-1</sup> or about half of the body average. Application of the single injection technique to the determination of interstitial space during sympathetic nerve stimulation has not been successful in the present study.

This study was supported by grants from the Swedish Medical Research Council (B75-40X-3514-04) and by the Karolinska Institutet, Stockholm.

## References

- BÄCKSTRÖM P. B., HOGG and A. MARTINSON, The sucrose space of human subcutaneous adipose tissue in obesity. *Acta med. scand.* 1966, 180, 123-127.
- DINTON, R. M., R. E. YORKEL and P. J. RANDALL, Measurement of concentrations of metabolites in adipose tissue and effects of insulin, alloxan-diabetes and adrenalectomy. *Biochem. J.* 1966, 100, 407-413.
- ENGELHARDT A., H. LIEBERMANN, Th. REUTER and K. ISACHSEN, Analyse der Wasserlöslichkeit und Lipidgehalt des menschlichen subcutanen Fettgewebes. *Z. klin. Chem. Biochem.* 1971, 19, 356-360.
- FREDHOLM, B. B. and A. FROMER, High energy phosphate compounds in adipose tissue: The effect of hemorrhage. *Acta physiol. scand.* 1974, 91, 165-171.
- GORESKY C. A., A linear method for determining liver sinusoidal and extravascular volumes. *Amer. J. Physiol.* 1963, 204, 626-640.
- HAMILTON, W. F., I. W. MOORE, I. M. KINGMAN and J. M. SPURLING, Studies on the circulation. IV. Further analysis of the injection method, and of changes in hemodynamics under physiological and pathological conditions. *Amer. J. Physiol.* 1932, 99, 334-351.
- LAMM, N. A. and P. SEARSD, Monoexponential extrapolation of tracer clearance curves in kinetic analysis. *Circulat. Res.* 1971, 29, 76-87.
- LAW R. O. and C. P. PHILLIPS, The size of the sucrose, raffinose, and inulin spaces in the gastrocnemius muscle of the rat. *J. Physiol. (Lond.)* 1966, 186, 547-557.
- MEIER, P. and K. L. ZIERLER, On the theory of the indicator-dilution method for measurement of blood flow and volume. *J. appl. Physiol.* 1954, 6, 731-744.
- ÖBERG, B. and S. ROSELL, Sympathetic control of consecutive vascular sections in canine subcutaneous adipose tissue. *Acta physiol. scand.* 1967, 71, 47-56.
- OWEN, C. A., *Diagnostic radioisotopes*. Charles C. Thomas, Springfield, USA 1959, p. 165.
- REINERT, E. M. and S. ROSELL, The influence of sympathetic adrenergic vasoconstrictor nerves on transport of diffusible solutes from blood to tissues in skeletal muscle. *Acta physiol. scand.* 1962, 54, 223-230.
- ROSELL, S., Release of free fatty acids from subcutaneous adipose tissue in dogs following sympathetic nerve stimulation. *Acta physiol. scand.* 1966, 67, 343-351.
- ZIERLER, W. H. and C. A. GORESKY, Transcapillary exchanges in the working left ventricle of the dog. *Circulat. Res.* 1971, 24, 181-207.
- ZIERLER, K. L., Theory of use of indicators to measure blood flow and extracellular volume and calculation of transcapillary movement of tracer. *Circulat. Res.* 1963, 12, 464-471.
- ZIERLER, K. L., Theoretical basis of indicator-dilution methods for measuring flow and volume. *Circulat. Res.* 1962, 10, 393-407.

Table I. Body weight and weight of heart, interscapular brown adipose tissue (ISBAT) and adrenals in control, cold-acclimated, trained and isoprenaline (ISO)-treated rats.

Weight	Control	Cold-acclimated	Trained	ISO-treated
Body wt, g	365 $\pm$ 9.4 <sup>a</sup> (17)	276 $\pm$ 9.9 <sup>a</sup> (9)	275 $\pm$ 10.9 <sup>a</sup> (7)	302 $\pm$ 9.3 (6)
Heart, mg	822 $\pm$ 17.3	954 $\pm$ 21.9 <sup>a</sup>	912 $\pm$ 15.1	1231 $\pm$ 22.5 <sup>a</sup>
ISBAT, mg	383 $\pm$ 11.5	823 $\pm$ 34.6	732 $\pm$ 58.0 <sup>a</sup>	513 $\pm$ 16.7 <sup>a</sup>
Adrenal, mg	67.7 $\pm$ 2.4	69.7 $\pm$ 1.7	68.6 $\pm$ 1.3	76.5 $\pm$ 1.5 <sup>a</sup>

Mean  $\pm$  S.E.

n = number of rats.

a = significant difference from the controls.

\*  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  (Student's t-test).

of all ( $p < 0.001$  Student's t-test). All the treatments used resulted in hypertrophy of the ISBAT tissue as is seen by its increased weight after the treatments. In the isoprenaline-treated rats the increase was smaller than that in the cold-acclimated ( $p < 0.001$ ) or trained ( $p < 0.005$ ). Only repeated injections of isoprenaline increased the weight of the adrenals.

heart enzymes

Table II shows that both the cold-acclimation and the swimming exercise used increased the activities of the aerobic enzymes of the myocardium. Mean DEH activity increased 41% with cold-acclimation and 40% with training. The activity of CS was increased 15% and 19% and that of MDH 23% and 20% in cold-acclimated and trained rats, respectively. By contrast, in isoprenaline-treated rats, the activity of CS was decreased by 29%. The decreases in the mean activities of MDH (-6%) or SDH (-14%) are not statistically significant. Of the anaerobic enzymes only HK showed significant changes in response to the treatments used. The HK activity of heart homogenate of the isoprenaline-treated rats was 35% higher than that of heart homogenate of the controls. Cold-acclimation and exercise in turn caused a 15% and a 10% increase over the control level, respectively. The increase in HK-activity in the isoprenaline-treated rats was significant.

Table II. Activities of cardiac muscle enzymes and protein content in control, cold-acclimated, trained and isoprenaline (ISO)-treated rats.

Enzyme	Control	Cold-acclimated	Trained	ISO-treated
Aspartate dehydrogenase	45.2 $\pm$ 3.37	63.5 $\pm$ 3.63 <sup>a</sup>	63.0 $\pm$ 4.4 <sup>a</sup>	40.3 $\pm$ 3.57
Lactate dehydrogenase	540 $\pm$ 16.4	642 $\pm$ 34.9 <sup>a</sup>	624 $\pm$ 19.0 <sup>a</sup>	525 $\pm$ 31.5
Isocitrate dehydrogenase	74.1 $\pm$ 1.33	85.0 $\pm$ 3.87 <sup>a</sup>	88.0 $\pm$ 4.95 <sup>a</sup>	52.4 $\pm$ 3.17 <sup>a</sup>
Malate dehydrogenase	5.91 $\pm$ 0.20	6.85 $\pm$ 0.53 <sup>a</sup>	6.35 $\pm$ 0.29 <sup>a</sup>	8.02 $\pm$ 0.41
Phosphoenolpyruvate carboxykinase	48.1 $\pm$ 2.86	42.8 $\pm$ 1.93	49.0 $\pm$ 6.40	40.3 $\pm$ 6.52
Lactate dehydrogenase	535 $\pm$ 28.7	639 $\pm$ 30.2	828 $\pm$ 131.8	636 $\pm$ 55.4
Protein	77.2 $\pm$ 2.89	87.0 $\pm$ 2.51	84.5 $\pm$ 2.62	64.1 $\pm$ 1.68 <sup>a</sup>

Enzyme activities are expressed as  $\mu$ moles of substrate utilized per hour per g wet weight, and protein content as mg protein in cell free homogenate from 1 g of muscle. Values are means  $\pm$  S.E. Numbers of rats are given in Table I.

\* Significant difference from the control:  $p < 0.05$ ,  $p < 0.02$ ,  $p < 0.01$  and  $p < 0.001$  (Student's t-test).

cold tolerance of rats, but does not extend swimming time (LeBlanc, Vallieres and Adam 1972). The present study was undertaken in order to compare the metabolic changes caused by strenuous physical training in the muscle with those caused by cold-acclimation and by repeated injections of isoprenaline.

## Material and methods

### Animals

Four groups of male albino rats of Wistar/Al/Han/Mal/Khan 67 strain weighing approximately 200 g at the beginning of the experiments were used in these studies. The first group consisted of control rats and the second group of cold acclimated rats (4°C for 5 weeks). The rats of the third group were trained by swimming for 1/2 h daily in water at 30°C. The swimming program was performed five times per week for 5 weeks. A weight of 5 g was attached at the base of the tail, and the weight was gradually removed during the first week to the final weight of 10 g. The rats of the fourth group received daily subcutaneous injections of isoprenaline (0.3 mg/kg) prepared fresh each day in an olive oil suspension. The rats were given five times per week for 5 weeks. The rats were killed by decapitation and hearts, adrenals and interscapular brown adipose tissues (ISBAT) were removed and weighed. The weights were adjusted to a common body weight by analysis of covariance.

### Enzyme assays

Samples of the gastrocnemius muscle and of the left ventricular muscle were immediately frozen in liquid nitrogen. The samples were stored frozen at -80°C until assayed. The muscle samples were then homogenized in a Potter Elvehjem glass homogenizer in Tris-HCl buffer (0.1 M, pH 7.4) to a 2% homogenate and centrifuged for 10 min at 1000 g at 4°C to remove unbroken cells and particulate debris. The supernatants were used for determination of the enzyme activities. Measurements are made at 25°C with a Cary 118 spectrophotometer equipped with a recorder.

Succinate dehydrogenase (SDH) activity was analysed according to Earl and Horner (1965), malate dehydrogenase (MDH) according to Ochoa (1955) and citrate synthase (CS) activity according to Sjöberg (1962). The activities of hexokinase (HK), phosphofructokinase (PFK) were determined according to Bergmeyer *et al.* (1969) and Boström *et al.* (1973), respectively. For the determination of lactate dehydrogenase (LDH) activity the standardized method of Biochemical Boehringer was used. All enzyme activities are expressed as  $\mu$ moles substrate utilized per min per g wet tissue weight. The protein contents of homogenates were estimated by the phenol method (Lowry *et al.* 1951).

### Cold tolerance and swimming time test

Cold tolerance and swimming time tests were made after the fourth week of treatment. The same rats were never used for both of the tests.

Cold tolerance was measured at -18°C. Four animals in a cage, each belonging to a different group, were placed at that temperature. Core temperatures were obtained with a thermocouple inserted to a depth of 4 cm.

The swimming time was measured in water at 30°C with a load of 5% of the body weight attached at the base of the tail. Animals in all four groups were used simultaneously in the test. The end point for swimming time was calculated when the rats remained more than 15 s under the water (LeBlanc *et al.* 1972).

## Results

### Body and organ weights

At the beginning of the experiments the body weights of the rats were identical in all experimental groups. At the end of the 5-week experimental period the control and isoprenaline-treated animals weighed significantly more than the cold-acclimated or exercised animals. Thus the cold-acclimated and the exercised rats gained less weight than did rats of the other groups (Table 1). However the hearts of the control rats were larger than those of the other groups, while the hearts of the isoprenaline-treated rats were



TABLE I. Body weight and weight of hearts, intrascapular brown adipose tissue (ISBAT) and adrenals in control, cold-acclimated, trained and isoprenaline (ISO)-treated rats.

Parameter	Control	Cold-acclimated	Trained	ISO-treated
Weight (g)	305 ± 9.4 (9) <sup>a</sup>	276 ± 9.9 <sup>a</sup> (9)	275 ± 10.9 <sup>a</sup> (7)	302 ± 9.3 (6)
Heart (mg)	822 ± 13.3	956 ± 21.9 <sup>a</sup>	911 ± 15.1	1211 ± 22.5 <sup>a</sup>
ISAT (mg)	383 ± 11.3	823 ± 34.6	732 ± 58.0 <sup>a</sup>	513 ± 16.7 <sup>a</sup>
Adrenal (mg)	67.7 ± 2.4	68.7 ± 1.7	68.6 ± 1.9	76.5 ± 1.5

mean ± S.E.

n = number of rats.

<sup>a</sup> = significant difference from the controls.

<sup>a</sup> = 0.05, <sup>b</sup> = 0.01 and <sup>c</sup> = 0.001 (Student's t-test).

weight of all (p < 0.001, Student's t-test). All the treatments used resulted in hypertrophy of the ISBAT tissue as is seen by its increased weight after the treatments. In the isoprenaline-treated rats the increase was smaller than that in the cold-acclimated (p < 0.001) or trained (p < 0.005). Only repeated injections of isoprenaline increased the weight of the adrenals.

#### Enzyme activities

The results in Table II show that both the cold-acclimation and the swimming exercise used increased the activities of the aerobic enzymes of the myocardium. Mean OAD activity increased 41% with cold-acclimation and 40% with training. The activity of SDH was increased 15% and 19% and that of MDH 23% and 20% in cold-acclimated and trained rats, respectively. By contrast, in isoprenaline-treated rats, the activity of CS was increased by 29%. The decreases in the mean activities of MDH (-6%) or SDH (-14%) were not statistically significant. Of the anaerobic enzymes only HK showed significant changes in response to the treatments used. The HK activity of heart homogenate of the isoprenaline-treated rats was 35% higher than that of heart homogenate of the controls. Cold-acclimation and exercise in turn caused a 15% and a 10% increase over the control value, respectively. The increase in HK-activity in the isoprenaline-treated rats was significant.

TABLE II. Activities of cardiac muscle enzymes and protein content in control, cold-acclimated, trained and isoprenaline (ISO)-treated rats.

Enzyme	Control	Cold-acclimated	Trained	ISO-treated
Isocitrate dehydrogenase	45.2 ± 3.37	63.5 ± 3.63 <sup>d</sup>	63.0 ± 4.24 <sup>c</sup>	40.3 ± 3.57
Malate dehydrogenase	540 ± 16.4	662 ± 34.9 <sup>a</sup>	624 ± 19.0 <sup>d</sup>	525 ± 31.5
Aspartate aminotransferase	74.1 ± 1.35	85.0 ± 3.87 <sup>a</sup>	88.0 ± 4.95 <sup>a</sup>	52.4 ± 3.17 <sup>d</sup>
Hexokinase	3.91 ± 0.20	6.85 ± 0.53 <sup>a</sup>	6.55 ± 0.29 <sup>a</sup>	8.02 ± 0.41
Phosphofructokinase	49.1 ± 2.86	42.8 ± 1.93	49.0 ± 6.40	40.3 ± 6.52
Lactate dehydrogenase	555 ± 28.7	639 ± 30.2	829 ± 131.8	634 ± 55.4
Protein	78.2 ± 2.89	87.0 ± 2.51	84.5 ± 2.62	64.1 ± 1.48 <sup>d</sup>

Enzyme activities are expressed as  $\mu$ moles of substrate utilized per min per g wet weight, and protein content as mg protein in cell free homogenate from 1 g of muscle. Values are means ± S.E. Numbers of rats are indicated with those given in Table I.

<sup>a</sup> = Significant difference from the controls: <sup>a</sup> = 0.05, <sup>b</sup> = 0.02, <sup>c</sup> = 0.01 and <sup>d</sup> = < 0.001 (Student's t-test).

TABLE III Activities of gastrocnemius muscle enzymes and protein content in control, cold-acclimated, trained and isoprenaline (ISO)-treated rats.

Enzyme	Control	Cold-acclimated	Trained	ISO-treated
Succinate dehydrogenase	6.35 ± 0.32	8.25 ± 0.50 <sup>b</sup>	10.15 ± 1.04 <sup>d</sup>	9.85 ± 0.54 <sup>d</sup>
Malate dehydrogenase	124 ± 5.94	165 ± 12.3	186 ± 9.4 <sup>d</sup>	171 ± 6.1 <sup>d</sup>
Citrate synthase	13.4 ± 0.58	15.8 ± 1.82 <sup>b</sup>	20.2 ± 1.24 <sup>d</sup>	19.6 ± 0.92 <sup>d</sup>
Hexokinase	1.09 ± 0.04	1.27 ± 0.04	1.26 ± 0.08 <sup>b</sup>	1.73 ± 0.12 <sup>d</sup>
Phosphofructokinase	16.1 ± 1.32	15.7 ± 0.92	13.9 ± 0.63	15.3 ± 0.88
Lactate dehydrogenase	865 ± 42.3	920 ± 34.0	861 ± 19.3	896 ± 34.1
Protein	68.1 ± 1.82	68.1 ± 2.76	66.6 ± 2.01	67.1 ± 1.09

Enzyme activities are expressed as  $\mu$ moles of substrate utilized per min per g wet weight, and protein content as mg protein cell free homogenate from 1 g of muscle. Values are means  $\pm$  S.E. Numbers of rats are identical with those given in Table I.

Significant difference from the control: <sup>a</sup>  $p < 0.05$ , <sup>b</sup>  $p < 0.02$ , <sup>c</sup>  $p < 0.01$  and <sup>d</sup>  $p < 0.001$  (Student's *t* test).

cantly greater than that in the cold-acclimated ( $p < 0.05$ ) or trained rats ( $p < 0.05$ ). The protein concentration of the cell-free homogenate of the myocardium was significantly higher (12%) the control concentration for the cold-acclimated rats. A similar tendency was observable for the trained animals ( $p < 0.1$ ). On the other hand, the heart homogenate from the isoprenaline-treated animals had a significantly lower protein concentration than the corresponding homogenate from other groups (-17% in comparison with the control).

**Gastrocnemius muscle** The results in Table III show that all the treatments used induced a rise in the activities of aerobic enzymes of gastrocnemius muscle. Mean activity of SDH increased 58%, mean activity of CS 50% and mean activity of MDH 50%, respectively with exercise. The corresponding increases for SDH, CS and MDH were 30, 35 and 33% with cold-acclimation, and 55, 46 and 39% with repeated injections of isoprenaline. Activity of HK increased 16, 17 and 59% in response to training, cold-acclimation and isoprenaline treatment, respectively. The increase in HK activity was significantly greater in the isoprenaline-treated rats than in the cold-acclimated ( $p < 0.01$ ) or trained rats ( $p < 0.05$ ). No changes were found in the activities of LDH and PFK. No changes were detected in the protein concentration of the cell-free homogenates of the gastrocnemius muscles.

#### Cold tolerance and swimming time tests

When placed at  $-18^{\circ}\text{C}$  in groups of 4 the rats were able to maintain their body temperature for several hours (Fig. 1). After 12 hours at this temperature the colonic temperature decreased significantly more in the control animals than in other groups ( $p < 0.01$ - $p < 0.05$ , Student's *t* test). The cold-acclimated animals, on the other hand, were much more tolerant to cold than the trained ( $p < 0.02$ ) or isoprenaline treated rats ( $p < 0.05$ ) as shown by a smaller decrease in colonic temperature.

The swimming time was tested at  $30^{\circ}\text{C}$  with a tail load of 5% of body weight. The results in Fig. 2 show that the training program used significantly increased the swimming time of these animals ( $p < 0.001$ , Mann-Whitney U-test). Also the cold-acclimated animals showed a slight improvement in resistance to exercise ( $p < 0.05$ ). However the isoprenaline treatment did not prolong the swimming time.

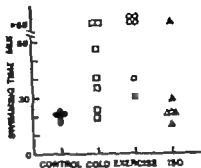
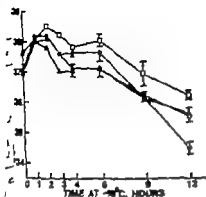


Fig. 2.

Fig. 1. Colonic temperature changes at  $18^{\circ}\text{C}$  of control ( $\bullet$ ), cold-acclimated ( $\square$ ), trained ( $\circ$ ) and isoprenaline-treated (daily injections of  $0.3 \mu\text{g/kg}$ ) rats ( $\Delta$ ). Each curve represents the mean  $\pm$  S.E. of 4 rats.

Fig. 2. Swimming time of control, cold-acclimated, trained, and isoprenaline-treated (ISO) rats with a tail of 5% of body weight in water at  $30^{\circ}\text{C}$ .

In the first swimming exercise at  $30^{\circ}\text{C}$  with a tail load of approximately 2% of body weight the mean colonic temperature of the rats had been fallen by  $5.28 \pm 0.34^{\circ}\text{C}$  with the 1st swim exercise. In the 18th swim with a tail load of approximately 3.5–4% of body weight a fall was significantly smaller ( $2.96 \pm 0.31^{\circ}\text{C}$ ,  $p < 0.001$ ).

### Discussion

The lower body weight and the higher heart weight of the exercised and cold-acclimated rats in this investigation agree with many previous reports of studies (e.g. Smith 1962, LeBlanc 1973, Hamilton and Ferguson 1972). An increase in the weight of ISBAT has been reported on cold-acclimated and on isoprenaline-treated rats (LeBlanc *et al.* 1972). In this study the swimming exercise used also increased the weight of ISBAT. This increase, however, may be due to repeated falls of body temperature caused by swimming in water at  $6^{\circ}\text{C}$ , and thus corresponds the cold-acclimation produced by repeated exposures of animals to cold (Tarkenton 1971). The very marked increase in the heart weight produced by repeated injections of isoprenaline has been reported earlier (Stanton, Brenner and Mayfield 1969, LeBlanc *et al.* 1972). This increase is due to a great extent to the increased water content of the heart tissue (Stanton *et al.* 1969) as is also seen by the decreased protein concentration of the heart muscle homogenates. In this study only the isoprenaline treatment increased the weight of the adrenals significantly. This could indicate that this treatment was the most stressful one. In contrast to findings in the literature (Fröberg, Östman and Sjöstrand 1972, Hearn and Vainio 1956, Smith 1962) the cold-acclimated or trained rats did not show any increase in adrenal weight. This may be due to the very heavy adrenals of the rat strain used, compared with values presented by other authors (e.g. Hearn and Vainio 1956, Fröberg *et al.* 1972).

Regular strenuous training increases the aerobic capacity of skeletal and cardiac muscle as is seen by the increased content of mitochondrial enzymes (Holloszy 1973). How similar changes in mitochondrial enzymes also are found with cold-acclimation (Hä 1963, Hamilton and Ferguson 1972). Accordingly in this study all the changes in aerobic enzymes in heart, as in skeletal muscle, were very similar in the trained as in the cold-acclimated rats. In the heart muscle these increases are due in part to increased protein content of the muscle, rather than to the increased enzyme activity as is seen by the lower protein concentration of heart muscle homogenate. Changes in the anaerobic enzymes in cold-acclimated and in trained rats also resemble each other whenever they are detected (Hannon 1962, Lamb *et al.* 1969, Hannon and Vaughan 1960, Goldrick *et al.* 1973). In this study significant changes (increase) were observable only in the activity of HK. No changes were detected both in heart and in skeletal muscle, and the changes were the same in both cold-acclimated and in trained rats. In addition, both these treatments significantly prolonged the swimming time and improved the cold resistance.

The results of LeBlanc *et al.* (1972) show that repeated injections of rats with isoprenaline improves the cold resistance. The present results show that this improvement can be accounted for by the increased heat production capacity of the skeletal muscle and brown fat, as is seen by the increase in the activities of aerobic enzymes in gastrocnemius muscle and the increased weight of ISBAT. On the other hand, the enzyme picture in heart muscle of isoprenaline-treated rats is very different from those of other groups. The decreased activity of the aerobic enzymes can be accounted for by the low protein concentration of the homogenate, due to increased water content of the cardiac tissue (Stanton *et al.* 1969). However, the fact that the ratio of anaerobic to aerobic enzymes is strongly anaerobic in the isoprenaline-treated rats, suggests that this treatment really shifts the heart metabolism to anaerobic pathways. It is known that isoprenaline reduces systemic and aortic blood pressure (and possibly reduces coronary perfusion pressure) at a time when heart rate and myocardial oxygen demands are maximally elevated. This could result in periods of myocardial hypoxia which could shift the metabolism of myocardium from aerobic to anaerobic pathways. Hypoxia has been suggested as one probable explanation of isoprenaline-induced cardiomegaly (Stanton *et al.* 1969), seen also in the present results. Moreover, the poor results of isoprenaline-treated rats in the swimming time test might be due to the failure of the heart.

As stated above, the metabolic changes in muscle metabolism as a result of strenuous training resemble very closely the changes produced by cold-acclimation or even by repeated injections of rats with isoprenaline. In addition, Kraus and Kirsten (1969) have suggested that corresponding enzymatic changes can be seen in mitochondria after thyroxine treatment as well. The possibility that the muscular shivering in cold corresponds to the muscular work of training and thereby is responsible for the metabolic changes found, can be excluded on the basis that shivering in the cold soon disappears (Smith 1962), and that similar metabolic changes can be found in cold-exposed poikilothermic animals which do not shiver (Lagerspetz, Harri and Oksalahti 1974). Furthermore, the metabolic changes induced by isoprenaline treatment or by hyperthyroidism do not require either muscular work or oxygen consumption. On the other hand, the swimming exercise used caused a marked fall in body temperature.

It is thus possible that the metabolic changes produced by the training program are a result of repeated falls in body temperature, rather than from muscular work per se. This possibility is improbable as the only reason, because very similar metabolic changes have been detected with strenuous exercise runs on a treadmill (Holloway 1967 Lamb 1969). Moreover Kraus and Kirsten (1969) found that a treadmill exercise induced metabolic changes in the myocardium and in the liver similar to those induced by a swimming exercise even in a much colder water temperature (25°C).

A conclusion: either increased muscular activity or cold are not necessarily required for metabolic changes of skeletal muscle. An alternate explanation is the increased metabolic stimulation by catecholamines. It is well known that the increased release of noradrenalin in the cold sensitizes the animals to the calorogenic action of this amine (Jansky 1973). It is also known that increased release of catecholamines is associated with physical training (Legendre 1971), and a suggestion has been made that trained athletes may be more sensitive to the metabolic effects of adrenalin and noradrenalin (Johnson and Rennie 1974). Repeated injection of rats with isoprenaline mimics the repeated stimulation of  $\beta$ -adrenoceptors by natural catecholamines, and also leads to sensitization of  $\beta$ -receptors (LeBlanc *et al.* 1972). Furthermore, the unpublished results from our laboratory (Harri and Tiiri) indicate that increased sympathetic activity or a mental stress (an electric shock connected with water drinking) can produce metabolic changes in the heart muscle, very similar to those described above. It is thus reasonable to assume that all the situations which include prolonged stimulation of adrenergic  $\beta$ -receptors result in intensification of tissue metabolism, mainly of aerobic pathways. It can be concluded that the metabolic changes induced e.g. by physical training or by cold acclimation are not necessarily specific indicators for these treatments.

That improved physical performance is associated with the metabolic changes induced by strenuous physical training is well known, but whether or not the metabolic changes resulting from other stresses demonstrate physical performance is another question. In the study cold acclimation prolonged the swimming time of rats. However the body temperature of the rats fell during the test. According to Dawson, Roemer and Horvath (1970) cold acclimation extends the swimming time by slowing the rate of body cooling. This may be the reason for the improved swimming time of the cold-acclimated rats in the present study. Unfortunately the ambient temperature also influences exercise tolerance in air in that cold-acclimated rats tolerate running exercise better at a cold temperature while control rats tolerate it better at room temperature (Dieter Altland and Highman 1969 1970), leaving the question of improved physical performance in cold-acclimated animals still unsolved. In accordance with the results of LeBlanc *et al.* (1972) the isoprenaline treatment did not extend swimming time despite the increased aerobic capacity of skeletal muscle. This demonstrates that the energy metabolism of muscle as such does not accurately give evidence about the physical fitness. This assumption is supported by the results of Bernard and Peter (1969), which show that although HK is very responsive to exercise, no relationship exists between HK activity of skeletal muscle and running endurance.

On the other hand, in the present results, cold tolerance was better in the trained rats

than in the controls. This improvement might be due to the repeated falls of body temperatures caused by the swimming. The results of the isoprenaline treatment, which did not induce changes in body temperature while increasing cold tolerance, indicate that neither cold environment nor a fall in body temperature are required for the improvement of cold tolerance. It can be concluded that all the situations which include intensification of base metabolism, mainly of aerobic pathways, result in improved cold tolerance. The fact that improved cold tolerance also results from treadmill exercise further supports this concept (Strømme and Hammel 1967).

Swimming is a generally used method in exercising rats. In these experiments the water temperature has been varied between 25°C and 33°C (Kraus and Kirsten 1969, 25°C; Hearn and Wainio 1956, 32°C; Arcos *et al* 1968, 33°C; Gould and Rawlinson, 1968, 27–30°C). All the temperatures used cause more or less marked changes in body temperature of rats (Dawson *et al* 1970). To what extent these falls have influenced the results obtained is not known. In future swimming experiments it is necessary to use a water temperature of 37°C (Dawson *et al* 1970) if the aim of the experiment is to measure the changes produced by physical activity only rather than those produced by cooling of the body and physical activity together.

This study was supported by the State Committee for Research in Sport and Physical Education in Finland through grants to one of the authors (J. V.). The authors wish to thank D. L. Nieminen of Reem Laboratories, Lääke and Medipolar Ltd., Turku for isoprenaline.

## References

- ADAMS, T. and E. J. HESBERLING, Human physiological responses to a standardized cold stress as modified by physical fitness. *J. appl. Physiol.* 1958, 13, 226–230.
- ARCOS, J. C., R. J. SOBAL, S.-C. SUN, H. F. AROUS and G. E. BURCH, Changes in ultrastructure and respiratory control in mitochondria of rat heart hypertrophied by exercise. *Exp. Mol. Pathol.* 1968, 49–65.
- BARRASFOVA, Z., *Acclimatization to Hypoxia and its Physiological Mechanisms*. Acad. Sci. USSR Moscow-Leningrad.
- BARNARD, R. J. and J. B. PETER, Effect of training and exhaustion on hexokinase activity of skeletal muscle. *J. appl. Physiol.* 1969, 27, 691–695.
- BASH, A., D. BRODICK, P. EYER, S. HOFER and B. PETER, Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Europ. J. Biochem.* 1969, 10, 193–206.
- BÖRTÉRM, S., M. FÄRLÉN, Å. HJALMARSSON and R. JOHANSSON, Activities of rat muscle enzymes after exercise. *Acta physiol. scand.* 1974, 90, 544–554.
- DAWSON, C. A., R. B. ROEMER and S. M. HOWE, Body temperature and oxygen uptake in sea and cold-adapted rats during swimming. *J. appl. Physiol.* 1970, 29, 150–154.
- DEITER, M. P., P. D. ALTLAND and B. HIGHMAN, Exercise tolerance of cold-acclimated rats. Serum liver enzymes and histological changes. *Canad. J. Physiol. Pharmacol.* 1969, 47, 1025–1031.
- DEITER, M. P., P. D. ALTLAND and B. HIGHMAN, Tolerance of unacclimated and cold-acclimated rats to exercise in the cold: serum, red and white muscle enzymes and histological changes. *Canad. J. Physiol. Pharmacol.* 1970, 48, 723–731.
- EARL, D. C. N. and A. KÖRNER, The isolation and properties of cardiac ribosomes and polyosomes. *Biochem. J.* 1963, 94, 721–
- FRÖBERG, S. O., I. ÖSTMAN and N. O. SÖSTRAND, Effect of training on esterified fatty acids and cholesteryl esters in adipose tissue in vitro. *Acta physiol. scand.* 1972, 86, 166–174.
- GOLLNICK, P. D., R. B. ARMSTRONG, B. SALTIN, C. W. SARRIS, J. W. L. SEMMOWICH and R. E. SELLERS, Effect of training on enzyme activity and fiber composition of human skeletal muscle. *J. appl. Physiol.* 1973, 34, 107–111.

- MCKE, P. D. and L. HERMANSON, Biochemical adaptations to exercise: Anaerobic metabolism. Pp. 1-10. In W. H. Jones, J. H. (ed.): *Exercise and sport sciences reviews*, Academic Press, New York-London, 1971, Vol. I.
- KILB, M. K. and W. A. RAWLINSON, Biochemical adaptation as response to exercise. I. Effect of running on the levels of lactic dehydrogenase, malic dehydrogenase and phosphorylase in muscles of 8- and 15-week-old rats. *Biochem. J.* 1970 73: 41-44.
- WASSERMAN, J., Role of circulating noradrenaline and adrenaline. Pp. 119-126. In Pernow P and Saltin, B. (eds): *Muscle metabolism during exercise*. Plenum Press, New York-London, 1971.
- KILB, M. K. and J. H. FERGUSON, Effects of exercise and cold acclimation on the ventricular and skeletal muscle of white mice (*Mus musculus*)—I. Succinate dehydrogenase activity. *Comp. Biochem. Physiol.* 1972 43A: 815-824.
- MCKE, I. P. Current status of carbohydrate metabolism in the cold-acclimated mammal. *Fed. Proc.* 1972 31: 836-841.
- MCKE, I. P. and D. A. V. GORMAN, Effect of prolonged cold exposure on the glycolytic enzymes of liver and muscle. *Amer. J. Physiol.* 1960, 192: 373-380.
- JONES, G. R. and W. W. WADEJO, Succinate dehydrogenase activity of the heart and skeletal muscle of several rats. *Amer. J. Physiol.* 1964, 196: 348-350.
- BRUNNEN, E. J. and T. ADAMS, Reactions of changing levels of physical fitness to human cold acclimatization. *J. appl. Physiol.* 1961, 16: 226-230.
- GILBERT, I. O., Biochemical adaptations in muscle. Effect of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J. Biol. Chem.* 1967 242: 2278-2282.
- GILBERT, I. O., Biochemical adaptations to anaerobic aerobic metabolism. Pp. 43-71. In Wilmore, J. H. (ed.): *Exercise and sport sciences reviews*, Academic Press, New York-London, 1973, Vol. I.
- GILBERT, I. O., L. B. ORSKOV, P. A. MOORE and L. J. DOW, Biochemical adaptation to endurance exercise in skeletal muscle. Pp. 51-64. In Pernow P. and Saltin, B. (eds): *Muscle metabolism during exercise*. Plenum Press, New York-London, 1971, Vol. II.
- CHICK, L., Non-shivering thermogenesis and its thermoregulatory significance. *Soc. Res.* 1973 48: 85-132.
- NEVILL, R. and M. REIDING, Athlete training and metabolism. *Nat. Sci.* 1974, 21: 585-587.
- ANDERSSON, W. R., The effect of repeated daily exposures to cold and of improved physical fitness on the metabolic and vascular responses to cold air. *J. Physiol. (Lond.)* 1961, 157: 209-220.
- WILKINSON, H. and R. KOSTER, Die Wirkung von körperlicher Training und des mitochondrialen Energieproduktion in Herzmuskel und im Leber. *Pflügers Arch. ges. Physiol.* 1970, 320: 334-347.
- ANDERSSON, K. Y. H. M. N. E. HARRIS and R. ORLANDINI, The role of the thyroid in the temperature regulation of the aquatic metabolism in the frog, *Rana temporaria*. *Gen. comp. Endocrin.* 1974, 22: 169-176.
- LEE, D. R., J. B. PETER, R. N. JEFFREY and H. A. WALLACE, Glycogen, lipids, and glyceraldehyde phosphate adaptations to exercise. *Amer. J. Physiol.* 1969, 217: 1628-1632.
- VAN AMBERGEN, K., Metabolic and circulatory aspects of tolerance to cold as affected by physical training. *Fed. Proc.* 1966 25: 1331-1334.
- BLANC, L., J. VALLEJOS and C. VACHON, Beta-receptor sensitization by repeated injections of isoproterenol and by cold adaptation. *Amer. J. Physiol.* 1972, 222: 1943-1946.
- SWARTZ, O. H. W. I. ROBINSON, A. L. FAIR and R. I. RANDALL, Protein measurement in the Folch plasma system. *J. Biol. Chem.* 1951, 193: 263-275.
- KILB, M. K. Malic dehydrogenase from pig heart. Pp. 734-739. Colowick, S. P. and M. Kaplan (eds): *Methods in enzymology*. Academic Press, New York-London, 1955, Vol. I.
- MCKE, I. P., The role of exercise in weight control. Pp. 103-123. In Wilmore, J. H. (ed.): *Exercise and sport sciences reviews*, Academic Press, New York-London, 1973, Vol. I.
- KILB, M. K. Cold acclimation—an altered steady state? *J. Amer. med. Ass.* 1962, 179: 948-954.
- JONES, P. A., Cellular respiration. Pp. 3-19. In Lewontin, L. M. (ed.): *Methods in enzymology*. Academic Press, New York-London, 1960, XIII.
- HUTTON, H. C., BURTON, G. and E. D. M. VERNON, Jr. Studies on isoproterenol-induced cardiorespiratory changes. *Amer. Heart J.* 1960, 77: 72-80.
- SCHMIDT, R. R. and H. T. HAMMER, Effect of physical training on tolerance to cold in rats. *J. appl. Physiol.* 1960, 22: 815-824.
- TAYLOR, H., Effect of repeated short-term exposures on brown adipose tissue of mice. *Am. Zool.* 1971, 11: 434-439.

## Secretion of Anterior Pituitary Hormones in Man: Effects of Ethyl Alcohol

By

J. LEPPÄLUOTO, M. RAPELI, R. VARIS and T. RANTA

Received 5 May 1975

### Abstract

LEPPÄLUOTO, J., M. RAPELI, R. VARIS and T. RANTA. Secretion of anterior pituitary hormones in man. Effects of ethyl alcohol. Acta physiol. scand. 1975. 95. 400-406.

The possibility that previously described effects of ethyl alcohol on peripheral endocrine glands are mediated via pituitary prompted this investigation on the effects of ethanol on anterior pituitary hormones. Nine healthy male subjects were given beverage containing ethanol (1.5 g/kg) or beverage alone per randomized cross-over study and plasma ACTH, FSH, GH, LH and TSH were measured by specific immunoassays up to 15 h and the urinary levels of adrenaline and noradrenaline by fluorometry. A blood LRP and TRP test was also carried out in a similar series of experiments. During the whole experiment there were no significant differences in the plasma levels of ACTH, FSH and TSH or in the urinary responses to LRF and TRP stimulation between ethanol treated and control subjects. Plasma FSH, LH and ACTH values were high (113-270 pg/ml) both in control and ethanol treated experiment suggesting that the subjects experienced apprehension toward the experiment. Plasma GH level exhibited a non-sleep related increase in the late evening (from 0.4 ng/ml at 6 p.m. to 3.1 ng/ml at 10 p.m.,  $p < 0.01$ ). This increase was not after alcohol ingestion ( $p < 0.01$ ). Plasma LH levels were significantly lower after 6 and 13 h in ethanol treated subjects than in controls (65 vs. 106 ng/ml,  $p < 0.01$  and 74 vs. 121 ng/ml,  $p < 0.05$  respectively). Because ethanol had no effect on the resting level of plasma GH or on the LH response to LRF we suggest that ethanol exerts these effects on a suprapituitary site.

Some behavioral effects of moderate doses of ethyl alcohol in man are regarded as stimulatory whereas most of the known endocrine effects appear to be inhibitory. Ethanol produces water diuresis accompanied by decreased chlorine output, similar to that seen after ingestion of water (Eggleston and Smith 1946). Uterine motility and milk ejection in response to suckling was abolished by ethanol in lactating rabbits (Fuchs and Wagner 1963). This all is suggestive of decreased release of vasopressin and oxytocin from hypothalamus.

There is evidence that alcohol might also inhibit the function of the pituitary-gonadal axis. Ovulation in response to electrical stimulation of hypothalamus was completely blocked in rabbits when alcohol was administered prior to stimulation (Saul 1959). In human subjects ethanol does not appear to change the plasma LH concentration (Toro *et al.* 1973), although testosterone concentration has been demonstrated to be greatly decreased during the heavy over period (Ylikahri *et al.* 1974).



and radioiodine uptake in chronic alcoholics and in experimental animals after long term treatment has been reported to be elevated (Murdock 1967), and the secretion of cortisol hormones increased after high or consecutive ethanol doses in man (Merry 1969, Mendelson *et al* 1971). The mechanism of these phenomena is unknown. In order to study the endocrine effects of ethyl alcohol might be mediated via the hypothalamus and the pituitary gland, we have studied the short term effects of ethanol on the plasma concentration of ACTH, FSH, GH, LH and TSH in healthy subjects.

# Material and Methods

21 healthy non-obese male medical students (21-25 yrs) volunteered for this study. None had history of chronic drinking and had not previously been exposed to repeated venipunctures or alcoholic studies. The experiments were carried out in 4 parts, 1-3 weeks apart from each other. Each experiment was begun at 6 p.m. on Friday and a light meal was allowed 2-4 h before. 1.5 g of ethanol per kg was administered diluted to 100-400 ml of beverage. Controls received beverage only. Alcohol or beverage was taken per os between 6 and 9 p.m.

Exp. I five randomly selected subjects (group A) are given the alcohol regimen and the remaining (group B) served as controls. Blood samples were drawn from each subject at 6, 8, 10 and 12 p.m. The subjects slept at the institute and on the following morning blood samples were taken at 7 and 9 a.m. Exp. II was carried out in the same manner as exp. I, but group B was given ethanol and group A served as controls.

Exp. III group A was given ethanol and group B served as controls as in exp. I, but the first blood sample was taken at 8-10 p.m. Then 100 µg of LH releasing factor (LRF) and 200 µg of TSH releasing factor (TRF) dissolved in 1 ml of saline were given. The peptides were synthesized by Ferring, Malmo, Sweden. Morning blood samples were taken 20 and 60 min later. Then the subject slept at their homes and brought a ml of morning urine to the institute in the following day.

Exp. IV was carried out as exp. III except that group B was given ethanol and group A served as controls. Urinary determinations. Blood samples are collected into heparinized test tubes and kept at 4°C for 1 h. The plasma was separated by centrifugation and kept at -20°C before the determinations (2-4 days later). The plasma ACTH and GH concentrations were estimated by commercial radioimmunoassays (Amersham, England and Kabi, Sweden). The materials for the radioimmunoassays for FSH and TSH were received from NIDDK, USA. The plasma ACTH concentrations are expressed as µU/ml and GH as µg/ml against 1st standards. FSH and LH are expressed as µg/ml of LER 907 standard and TSH as µU/ml of MRC standard 63/46. All the estimations were made in duplicate and in one run in order to avoid inter-assay variability (except ACTH). The intra-assay coefficient of variation calculated on all the duplicate estimations varied from 4 to 18% in different assays. The inter-assay coefficient of variation in ACTH radioimmunoassay was 4.3% at the dose level of 300 pg/ml (N=4). Hormone concentrations were read graphically from standard curves with percentage binding plotted against the logarithm of dose.

15 ml of 6 N HCl were added to 100 ml samples of urine and kept at -20°C before the estimation. Noradrenaline and adrenaline concentrations were estimated after aluminium oxide adsorption in fluorometry (Lundvall *et al*). Statistical comparisons between results obtained in various experimental groups were performed using computer program built up by Sakur and Gulliksen (1962). In brief, homogeneity of variance within each treatment was first tested according to Bartlett. In the absence of significant heterogeneity Duncan test for multiple comparisons are used, in other case non-parametric test of Mann-Whitney. P values smaller than 0.05 were regarded as significant.

# Results

General findings. All the test subjects experienced some apprehension toward venipunctures and their ability to tolerate the effects of ethanol, especially in first experiments. The following behavioral changes were observed after ingestion of ethanol: rapid and loud speaking, irritability, agitation, attempts to leave the institute. One subject in the first experiment experi-

## Secretion of Anterior Pituitary Hormones in Man, Effects of Ethyl Alcohol

By

J. LEPPÄLUOTO, M. RAPELL, R. VARIS and T. RANTA

Received 5 May 1975

---

### Abstract

---

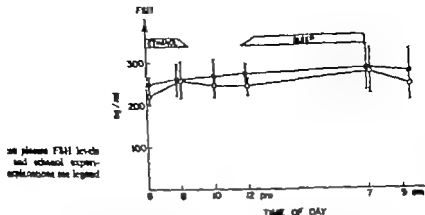
LEPPÄLUOTO, J., M. RAPELL, R. VARIS and T. RANTA. Secretion of anterior pituitary hormones in man. Effects of ethyl alcohol. *Acta physiol. scand.* 1975, 95, 400-406.

The possibility that previously described effects of ethyl alcohol on peripheral endocrine glands is mediated via pituitary prompted this investigation on the effects of ethanol on anterior pituitary. Nine healthy male subjects were given beverage containing ethanol (1.5 g/kg) or beverage alone in a randomized cross-over study and plasma ACTH, FSH, GH, LH and TSH were measured by specific immunoassays up to 15 h and the urinary levels of adrenaline and noradrenaline by fluorometry. A blinded LRF and TRF test was also carried out in a similar series of experiments. During the whole experiment there were no significant differences in the plasma levels of ACTH, FSH and TSH or in the urinary responses to LRF and TRF stimulation between ethanol treated and control subjects. Plasma FSH, LH and ACTH values were high (113-270 pg/ml) both in control and ethanol experiment suggesting that all subjects experienced apprehension toward the experiment. Plasma GH level exhibited a dose-sleep related increase in the late evening (from 0.4 ng/ml at 6 p.m. to 3.1 ng/ml at 10 p.m.,  $p < 0.01$ ). This increase was less after alcohol ingestion ( $p < 0.01$ ). Plasma LH levels were significantly lower after 6 and 13 h in ethanol treated subjects than in controls (65 vs. 106 ng/ml,  $p < 0.01$  and 74 vs. 121 ng/ml,  $p < 0.05$  respectively). Because ethanol had no effect on the resting level of plasma GH or on the LH response to LRF we suggest that ethanol exerts these effects on a suprapituitary site.

---

Some behavioral effects of moderate doses of ethyl alcohol in man are regarded as stimulatory whereas most of the known endocrine effects appear to be inhibitory. Ethanol produces water diuresis accompanied by decreased chlorine output similar to that seen after ingestion of water (Eggleton and Smith 1946). Uterine motility and milk ejection in response to suckling was abolished by ethanol in lactating rabbits (Fuchs and Wagner 1963). This all is suggestive of decreased release of vasopressin and oxytocin from hypothalamus.

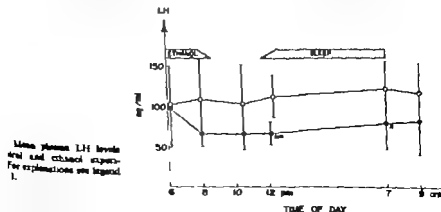
There is evidence that alcohol might also inhibit the function of the pituitary-gonadal axis. Ovulation in response to electrical stimulation of hypothalamus was completely blocked in rabbits when alcohol was administered prior to stimulation (Saul 1959). In human subjects ethanol does not appear to change the plasma LH concentration (Toro *et al.* 1973), although testosterone concentration has been demonstrated to be greatly decreased during the hangover period (Ylikahri *et al.* 1974).



246 to 277 ng/ml in ethanol treated subjects and no significant diurnal or alcohol were seen (Fig. 3). In LRF and TRF test plasma FSH presented a significant rise (Duncan's test) at 20 and 60 min both in control and ethanol treated subjects but the difference between the rises was not significant (Fig. 6 a).

**LH concentration.** Plasma LH in control subjects varied from 104 to 121 ng/ml in the experiment and did not show any diurnal fluctuation (Fig. 4). In ethanol treated LH levels were generally lower from 8 p.m. and the decrease was significant at 7 a.m. (65 vs. 106 ng/ml,  $p = 0.01$  and 74 vs. 121 ng/ml,  $p = 0.05$  Wilcoxon test). In TRF stimulation plasma LH rose in 20 min from 100 to 245 ng/ml in controls (Duncan test) and from 90 to 297 ng/ml ( $p = 0.01$ ) in alcohol treated subjects. These responses to LRF were similar in control and alcohol treated subjects ( $p > 0.05$ ).

**TSH concentration.** Plasma TSH varied from 3.7 to 4.3  $\mu$ U/ml in control and from 4.4  $\mu$ U/ml in ethanol treated subjects. No significant diurnal or ethanol effect was seen ( $p > 0.05$  Duncan test). In TRF stimulation test TSH rose in 20 min from 4.5 to 10.1  $\mu$ U/ml ( $p = 0.01$ ) in control and from 5.4 to 16.8  $\mu$ U/ml ( $p = 0.01$ ) in ethanol treated



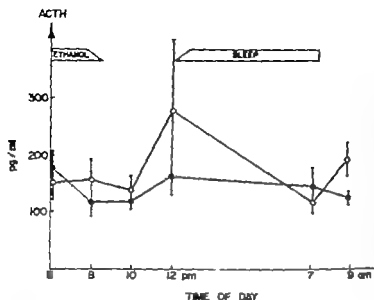


Fig. 1 Mean plasma ACTH levels of 9 normal men in control (open) and ethanol (black circle) experiment. Vertical bars denote  $\pm 1$  S.E. The asterisks and control circles indicate values significantly (one:  $p < 0.05$ , two:  $p < 0.01$ ) different from the respective value, aside ethanol circles significantly different than the respective control also. The absence of the asterisk denotes no significant differences ( $p > 0.05$ ).

enced nausea and vomiting. On the following morning subjective expressions of the hangover were present.

**Plasma ACTH concentration.** From the beginning of the experiment the plasma ACTH levels were high both in control and ethanol treated subjects: the respective mean values varied from 124 to 270 pg/ml and from 113 to 170 pg/ml, (Fig. 1). Variances were heterogeneous (Bartlett), and in non-parametric test (Wilcoxon) no significant differences between control and ethanol treated subjects were found.

**Plasma GH concentration.** In control subjects plasma GH rose from  $0.4 \pm 0.1$  ng/ml (mean  $\pm$  S.E.) at 6 p.m. to  $3.1 \pm 1.3$  ng/ml at 10 p.m. ( $p < 0.01$ , Wilcoxon test), and then returned to initial levels (Fig. 2). In ethanol treated subjects the plasma GH level varied from 0.4 to 0.8 ng/ml during the whole experiment and was significantly ( $p < 0.01$ ) lower at 10 p.m. than in controls at 10 p.m. ( $0.6$  vs.  $3.1$ ).

**Plasma FSH concentration.** Mean plasma FSH varied from 218 to 264 ng/ml in control

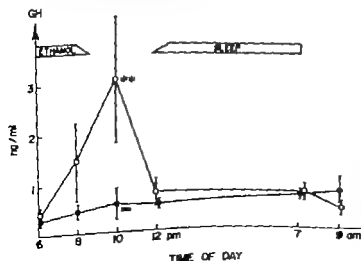


Fig. 2 Mean plasma GH levels in control and ethanol experiment. For explanations see legend in Fig. 1.

found to block ovulation (Saul 1958) as well as the late decrease of plasma testosterone as by Ylikahri *et al.* (1974). It is noteworthy that we did not find diurnal fluctuation in LH. It is possible that we have missed the LH discharges reported to occur in the morning (Nankin and Troen 1972).

There is no previous information about the effects of ethanol on plasma FSH and, according to our results, alcohol does not seem to have any effect on plasma FSH up to 15 h. Results also failed to reveal diurnal fluctuation in plasma FSH levels.

Our combined LRF and TRF test plasma LH and FSH responses to LRF were similar to control experiments. This suggests that the effects of ethanol on inhibition of secretion, described here, occurs at a suprapituitary site.

Control subjects exhibited a non-sleep related burst of GH in the evening (at 10 p.m.). A nocturnal rise in GH secretion generally occurs during the early sleep (Quabbe *et al.*

1968) but according to our results, GH is also secreted in the late evening in wakeful subjects. This finding was confirmed by a recent work (Plotnick *et al.* 1975). The observed rise of GH was however not seen after ethanol administration. Thus alcohol seems to inhibit the stimulated GH secretion, but has no effect on resting levels. Our finding is in agreement with that made by Arky and Finkel (1964), who demonstrated normal GH responses to ethanol induced hypoglycemia in 2 patients.

In the present study plasma TSH levels showed no diurnal fluctuation and were similar to control and ethanol experiments as were the responses to TRF. In previous studies there was no information about plasma TSH levels, but elevated radiolabeled thyroglobulin has been observed in chronic alcoholics and in rats after long term ethanol treatment (Murdock 1972). These results suggest that alcohol might have a direct stimulatory effect on the thyroid, and possible changes in plasma TSH might occur in late phase.

Previous reports about the effects of ethyl alcohol on the pituitary-adrenocortical axis concern the glucocorticoidal responses only. Non-alcoholic individuals respond to high doses ethanol with a rise of plasma cortisol (Merry and Marks 1969). After moderate alcohol doses, Kravitz *et al.* (1958) and Perlman *et al.* (1961) did not find adrenocortical stimulation in a short-time experiment. On the other hand, long-term alcohol ingestion is associated with adrenocortical activation, which is at the greatest during vegetative symptoms, such as gastric pains (Mendelson *et al.* 1971). In the present study the plasma ACTH concentrations were clearly elevated at the beginning of the experiments, both in control and ethanol runs. These high plasma ACTH levels were evidently due to enhanced emotional tension of the test subjects, who were for the first time exposed to this type of an experiment. Increased cortisol secretion in control experiments has been earlier observed in "first-time" participants by Spabum *et al.* (1958) and by Sutton and Casey (1975). This anxiety reaction interfered with the interpretation of our ACTH results in this respect. We emphasize that ethanol administration did not produce a tranquillizing effect that would be reflected in the fall of ACTH secretion.

Collection of urine samples for measurement of adrenaline and noradrenaline secretions was carried out after the shorter and later experiments, when the anxiety reactions were absent. The urine levels of adrenaline and noradrenaline were normal and similar both in control and ethanol experiments. In previous work, increased urinary adrenaline secre-

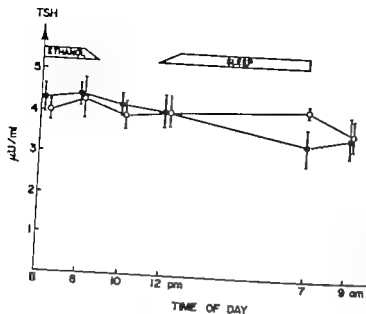


Fig. 5. Mean plasma TSH levels in control and ethanol-treated subjects. For explanations see legend in Fig. 1.

subjects (Fig. 5 and 6 c). The increases in responses to TRF were again similar in control and ethanol-treated subjects ( $p > 0.05$ ).

**Urine catecholamine concentrations** Urinary noradrenaline level in control subjects was  $37 \pm 3$  ng/ml at 7 a.m. and adrenaline level  $4.1 \pm 0.7$  ng/ml. After ethanol administration the respective noradrenaline and adrenaline levels were  $38 \pm 2$  and  $3.2 \pm 1.4$  ng/ml ( $p > 0.05$ ).

### Discussion

The most prominent effect of alcohol in the present study was the decrease of plasma LH after 6 h from the beginning of the experiment. Toro and his coworkers (1973) failed to find any changes in plasma LH levels after ethanol administration. This is not necessarily in disagreement with our results, because in latter study the observation period was only 3 h. However, in blood samples obtained 12 h after ethanol ingestion a significant decrease in plasma testosterone with a compensatory rise in plasma LH was recently demonstrated (Ylikahri *et al.* 1974). At the moment we cannot give an explanation for this divergence. Our low LH values are in better agreement with the previous animal studies, in which ethanol

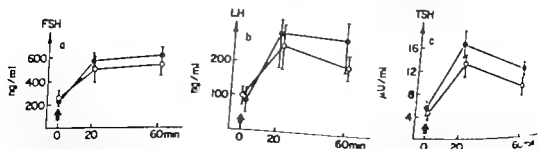


Fig. 6. Mean plasma FSH (6 a), LH (6 b) and TSH (6 c) levels in control and ethanol-treated subjects. Open circle = control experiment and black circle = ethanol experiment. The arrow indicates the time of TRF administration.

All the increments after 20 min significant ( $p < 0.01$ ), but the differences between control and ethanol experiment not ( $p > 0.05$ ).

## Uptake of Vitamin D<sub>3</sub> in the Mouse and the Quail

By

LENNART DENCKER and HANS TÄLLVÉ

Received 6 May 1975

### Abstract

L. and H. TÄLLVÉ, *Studies on the renal uptake of vitamin D<sub>3</sub> in the mouse and the quail*. Acta physiol. scand. 1975, 95, 407-416.

Uptake of labelled vitamin D<sub>3</sub> was studied in the mouse and the quail. Upon the administration of vitamin D<sub>3</sub> autoradiographic experiments showed specific accumulation of radioactivity in renal tubules of the mouse kidney. This was still obvious 18 days after the administration. On the other hand, the uptake in the kidney did not exceed the level of the blood. In the mouse there was no increase in the amount of steroid in the kidney after the injection of vitamin D<sub>3</sub>, maximum reached 24 hours after the administration. The amount of steroid which accumulated in the kidney was proportional to the injected dose of the vitamin—from doses at 4.5 µg to 4.5 mg. Cellulose Sephadex showed that most of the renal vitamin D<sub>3</sub> is present in non-metabolized form. Cellular studies showed that most radioactivity in the kidney was present in the microsomal and macrovesicular fractions. Upon separation of the fractions most radioactivity was still bound to these particles.

of Vitamin D<sub>3</sub>, kidney, proximal tubule, autoradiography, mouse, quail

In recent years indicate that the kidney plays an important role in the metabolism of vitamin D to more active metabolites, (for a review see e.g. Rodanek 1974). In addition the kidney is considered to be an important site of action for the vitamin, although most of its effects are still obscure (Gekke *et al.* 1971, Paschert *et al.* 1972 a, b).

In a preliminary autoradiographic study a very selective accumulation of radioactivity was observed in the kidney after the administration of labelled vitamin D<sub>3</sub> in mice (Dencker & Tällvé 1973). In the present study more data on the renal uptake of vitamin D<sub>3</sub> are presented. Mice and quails are used as experimental animals.

### Material and methods

<sup>3</sup>H-cholecalciferol (<sup>3</sup>H-vitamin D<sub>3</sub>), spec. activity 32.2 mCi/mmol, (1α, 25-<sup>3</sup>H)-cholecalciferol (<sup>3</sup>H-1,25-D<sub>3</sub>), spec. activity 8.2 Ci/mmol and (26 (27-<sup>3</sup>H)-25-hydroxycholecalciferol (<sup>3</sup>H-25-hydroxyvitamin D<sub>3</sub>), spec. activity 6.9 Ci/mmol were obtained from the Radiochemical Centre, Amersham, England.

Mice of the C57BL strain (body weight 20-25 g) were used. The mice were fed either a complete diet or vitamin D-deficient rachitogenic pellet diet of composition similar to that described by

tion after ethanol ingestion has been reported at 4 h (Perman 1958). Our urine collection occurred at 15 h, and hence, the comparison between his and our results is difficult.

Our subjective observations suggest that administration of moderate doses of ethanol may have stimulated behaviour in healthy male subjects. In addition, ethanol resulted in decreased secretion of LH and GH but did not effect on the secretion of FSH, TSH, adrenaline or noradrenaline. A normal response of LH to LRF and a normal resting level of GH suggests that ethanol exerts this inhibitory effect on hypothalamic or suprahypothalamic structures.

This study was supported by a grant from the Alcohol Research Foundation, Finland. We are grateful to the National Pituitary Agency NIAMDD U.S.A. for providing material for human FSH, LH and TSH radioimmunoassays. We also thank Dr Jan Mulder for supplying synthetic LRF and TRF (Ferring Ltd, Sweden).

References

ARNEY, R. A. and N. FREINKEL, The response of plasma human growth hormone to insulin and ethanol-induced hypoglycemia in two patients with isolated adrenocorticotrophic defect. *Diabetologia* 1964 11: 547-550.

ECCLISTON, M. G. and I. G. SMITH, The effect of ethyl alcohol and some other diuretics on chloride excretion in man. *J. Physiol. (Lond.)* 1946, 104: 435-442.

FUCHS, A. R. and G. WAGNER, The effect of ethyl alcohol on the release of oxytocin in rabbits. *Acta endocrinologica (Kbh.)* 1963 44: 593-603.

KRUMH, F. E., A. O. VARTIA and O. FORSANDER, Experimentelle Studien über die biologische Wirkung von Alkohol. II. Alkohol und Nebennierenrunden-Funktion. *Ann. Med. et Pharm.* 1948, 36: 44-49.

MENDELSON, J. H., M. OGA and N. K. MELLO, Adrenal function and alcoholism. I. Serum cortisol. *Psychosom. Med.* 1971 33: 145-157.

MERLEY, J. and V. MARKS, Plasma-hydrocortisone response to ethanol in chronic alcoholics. *Lancet* 1964 i: 921-923.

MURDOCK, H. R. J., Thyroidal effect of alcohol. *Quart. J. Stud. Alc.* 1967 28: 419-421.

NANKIN, H. R. and P. TROEN, Overnight patterns of serum luteinizing hormone in normal men. *J. Clin. Endocr.* 1972 35: 705-710.

QUARRE, H. J., E. SCHILLING and H. HALGE, Pattern of growth hormone secretion during a 4-hour fast in normal adults. *J. Clin. Endocr.* 1966, 26: 1173-1177.

PERMAN, E. S., The effect of ethyl alcohol on the secretion from the adrenal medulla in man. *Acta physiol. scand.* 1958, 44: 241-247.

PERMAN, E. S., Observations on the effect of ethanol on the urinary excretion of histamine, 5-hydroxyindoleacetic acid, catecholamines and 17-hydroxycorticosteroids in man. *Acta physiol. scand.* 1961 31: 6-29.

PLOTNICK, L. P., R. G. THOMPSON, A. KOWARSKI, L. deLACROIX, C. J. MASON and R. M. BLIZZARD, Circadian variation of integrated concentration of growth hormone in children and adults. *J. Clin. Endocr.* 1975, 40: 240-247.

SARIZ, E. and R. GUILLEMIN, Utilisation de routine d'un grand ordinateur dans l'analyse mathématique des résultats en biologie expérimentale. *C. R. Acad. Sci. (Paris)* 1962, 254: 3743-3745.

SAMMUN, M., D. HAMBURG, R. GRUNER, H. PERLEY, H. BASOWITZ, S. KORCHIN and J. CHEVALIER, Superficial of preexperimental studies: the psychosomatic laboratory. *Arch. Neurol. Ps. Inst. (Chic.)* 1957 78: 207-219.

SAUL, G., Blockade of ovulation in the rabbit by toxic doses of ethyl alcohol. *Am. Rev.* 1959, 122: 332.

SUTTON, J. R. and J. H. CASEY, The adrenocortical response to competitive athletics in elite athletes. *J. Clin. Endocr.* 1975, 40: 133-138.

TORO, G., R. C. KOLODNY, L. S. JACOBS, W. H. MASTERS and W. H. DAUGHADAY, Failure of alcohol to alter pituitary and target organ hormone levels. *Clin. Res.* 1973 21: 505.

YLIKAHRI, R., M. HUTTUNEN, M. HÄRKÖNEN, U. SEUDERLING, S. ONKKA, E. L. KA. OLIN and H. ADOLF-CREUTZ, Low plasma testosterone values in men during hangover. *J. Ster. Biochem.* 1974, 3: 435-437.



3-phase was then separated after adding 2 volumes of water. The aqueous methanol residue was further dried and with 1 volume of chloroform. The combined chloroform-phases were evaporated to dryness and immediately redissolved in 0.2 ml *n*-hexane-chloroform 35:65 (v/v) to be used for chromatography. The column chromatography was performed at 4°C with Sephadex LH-20 and from Pharmacia Fine Chemicals, Uppsala, Sweden (Hsieh and DeLuca 1971) using column equilibrated with *n*-hexane-chloroform 35:65 (v/v). The same solvent system was used for elution and fractions were collected. The fractions were evaporated with air to dryness in liquid scintillation vials as packed liquid scintillation spectrometer after the addition of scintillation fluid (0.25 g PPO/g ter solvent). Correction for quenching was made by the use of an external standard. <sup>3</sup>H-vitamin D<sub>3</sub>, <sup>14</sup>C-vitamin D<sub>3</sub> and [<sup>3</sup>H]-25-hydroxyvitamin D<sub>3</sub> were used as references to detect their elution changes.

#### Counting

Determination of radioactivity in the kidney after the injection of <sup>3</sup>H-vitamin D<sub>3</sub> as determined in a scintillation counter. Each mouse in the first series received 0.1 µCi (4.8 ng = 1.22 × 10<sup>-10</sup> mol) <sup>3</sup>H-vitamin D<sub>3</sub> dissolved in 25 µl ethanediol. In the other series 0.1 µCi <sup>3</sup>H-vitamin D<sub>3</sub> as mixed with unlabeled vitamin D<sub>3</sub> and injected (dissolved in 25 µl ethanediol). Thus in the second series each mouse received 4.8 ng = 1.22 × 10<sup>-10</sup> mol vitamin D<sub>3</sub> in the third series 0.48 µg = 1.22 × 10<sup>-9</sup> mol vitamin D<sub>3</sub> fourth series 4.8 µg = 1.22 × 10<sup>-8</sup> mol vitamin D<sub>3</sub> and in the fifth series 48 µg = 1.22 × 10<sup>-7</sup> mol vitamin D<sub>3</sub>. Mice in each series were killed respectively 1 h, 2 h, 4 h, 8 h, 4 h and 48 h after the injection of <sup>3</sup>H-vitamin D<sub>3</sub>. They were perfused as described above (see under Column chromatography) and their kidneys removed. These were then dissolved in Soluene and the radioactivity as determined by scintillation counting. With the knowledge of the specific activity of the parent vitamin the disintegrations per minute in the samples were converted to the absolute amount (pmol) of steroid in the kidney.

#### Isotachopheresis

Animals fed the vitamin D-deficient diet were injected i.p. with 0.1 µCi (4.8 ng) <sup>3</sup>H-vitamin D<sub>3</sub> (dissolved in 25 µl dimethylsulfoxide). Six mice were killed at 24 h and six at 6 days after having first been fed. 12 mice were injected in the same way with 0.1 µCi <sup>3</sup>H-vitamin D<sub>3</sub> together with 4.8 µg non-radioactive vitamin D<sub>3</sub> and were killed at the same survival intervals as above (6 mice at each interval) after 8 hours post-injection. The kidneys were removed and manually homogenized in 10 ml 0.25 M sucrose in a shock glass tissue grinder with teflon pestle (Kontes Ltd., NJ USA). The tissue suspensions were filtered through double layer of gauze. Cellular fractionation as performed by centrifugation in 0.25 M sucrose in a Beckman L5-50 high speed centrifuge (Beckman Ltd., London, England) first at 630 g for 10 min to obtain the nuclear fraction, then at 14,000 g for 20 min to obtain the mitochondrial fraction and finally 50,000 g for 1 h to obtain the microsomal and supernatant fractions (see 1097 text). Microscopic examination of the nuclear fraction showed that it was practically free of contamination with broken cells and debris. The radioactivity of the fractions was determined by liquid scintillation counting after dissolution in Soluene as described above.

In additional experiments two mice were injected i.p. with 0.1 µCi <sup>3</sup>H-vitamin D<sub>3</sub> together with 4.8 ng unlabeled vitamin D<sub>3</sub> and were killed after 24 h. The kidneys were removed after perfusion and the nuclear and mitochondrial fractions were isolated as described above. These fractions were then diluted with 5 ml sucrose and subjected to sonication in a Branson Sonifier at 5A for 4–2 min (interrupted to avoid heating) in an ice bath. The sonified fractions were then centrifuged at 103,000 g for 1 h and the radioactivity in the resulting pellet and supernatant were determined by liquid scintillation counting as described above.

## Results

### Whole body autoradiography

After the injection of <sup>14</sup>C-vitamin D<sub>3</sub> there was no difference in the distribution of radioactivity between the mice fed the complete diet and those which received the vitamin deficient diet. At the studied survival intervals there was nor an obvious difference between the i.v. and the s.c. injected animals. In the description below no distinction will be made between the different groups of animals.

Stenbock and Black (1925) and Numerof *et al.* (1935). The diets were supplied by AB Astra-Evo, Södertälje, Sweden. The vitamin D-deficient diet consisted of (per cent of weight): corn (ground) 75, wheat gluten 20,  $\text{CaCO}_3$  3, NaCl 1 and a vitamin mixture 1. The vitamin mixture had the following composition (per cent of weight):

Vitamin A 500 000 IE/g 0.200, thiamine 0.040, riboflavin 0.124, pyridoxine 0.050, Ca-pantothenate 45% 0.222, nicotinic acid 0.400, vitamin B<sub>12</sub> 0.05% 0.400, vitamin K (menadiolone) 0.015, biotin 1.000, ascorbic acid 5.000, inositol 0.300,  $\alpha$ -tocopherol 500 mg/g 0.840, choline chloride 50% 20.00, p-aminobenzoic acid 2.500, folic acid 0.005 and starch (protein-free) 69-604. The content of calcium and phosphorus in the diet was 1.16% and 0.27% respectively. The mice fed this diet received it from their third week of age until used in the experiments 3-8 weeks later. The serum calcium value of the mice fed the vitamin D-deficient diet was  $8.4 \pm 0.5$  mg/ml (mean  $\pm$  S.E., 7 determinations), while in the mice receiving the complete pellet diet the serum calcium concentration was  $9.7 \pm 0.2$  mg/ml (mean  $\pm$  S.E., 7 determinations). The serum calcium was determined by a flame photometric method at the Department of Clinical Chemistry, University Hospital, Uppsala.

*Quails.* One male and one female Chinese dwarf quail (*Excalfactoria chinensis*, b.w. about 40 g) and a laying Japanese quail (*Coturnix coturnix japonica*, b.w. about 140 g) were used. The birds were fed a complete pellet diet (Harald Fors & Co AB, Stockholm, Sweden).

### *Autoradiography*

*Whole body autoradiography.* Mice fed the complete diet, mice fed vitamin D-deficient diet and the quails were used in this investigation. The mice received <sup>14</sup>C-vitamin D<sub>3</sub> either subcutaneously in the rear (dissolved in 25  $\mu$ l dimethylsulfoxide) or intravenously in a tail vein (dissolved in 25  $\mu$ l absolute ethanol). The quails received the vitamin subcutaneously dissolved in 25  $\mu$ l dimethylsulfoxide. In the complete diet group three male mice were injected i.c. receiving 1.6  $\mu$ Ci (19  $\mu$ g) <sup>14</sup>C-vitamin D<sub>3</sub> each and then killed at 5 min, 4 h and 24 h. Five mice were injected s.c., each animal receiving 1.0  $\mu$ Ci (12  $\mu$ g) <sup>14</sup>C-vitamin D<sub>3</sub> and were then killed after 1 h (male), 4 h (male), 8 h (male), 4 days (female) and 18 days (female). Ten mice fed the vitamin D-deficient diet were injected s.c. receiving 0.5  $\mu$ Ci (6  $\mu$ g) <sup>14</sup>C-vitamin D<sub>3</sub> each and were killed after 1 h (2 male mice), 8 h (2 male mice), 4 h (2 female mice), 4 days (1 female mouse), 8 days (2 female mice) and 18 days (1 female mouse). The Chinese dwarf quails were injected s.c. receiving 1.0  $\mu$ Ci (12  $\mu$ g) <sup>14</sup>C-vitamin D<sub>3</sub> each and were killed after 8 h (male) and 4 h (female). The Japanese quail was injected s.c. with 3.0  $\mu$ Ci (36  $\mu$ g) <sup>14</sup>C-vitamin D<sub>3</sub> and was killed after 4 days.

At the stated survival intervals the animals were anesthetized with ether, embedded in an aqueous solution of carboxymethyl cellulose, immersed in a mixture of solid CO<sub>2</sub> and hexane ( $-78^\circ\text{C}$ ) and subjected to autoradiography according to Ullberg (1954-1958). This procedure includes sectioning of the whole animals (20  $\mu$ m thick sections) at  $-15^\circ\text{C}$  in a microtome of the type (No 800, Minnesota Mining and Manufacturing Co. USA), freeze-drying of the sections at  $-15^\circ\text{C}$  and autoradiography by apposition of the sections against X-ray films (Structurix D7, Gevaert). The exposure (the exposure time being 8-28 months) was carried out at  $-15^\circ\text{C}$  to avoid diffusion of lipid soluble material. After the exposure time the sections were separated from the films. The films were developed in Gevaert G230 and fixed in Gevaert G305A.

*Microautoradiography.* One mouse, fed the vitamin D-deficient diet was injected subcutaneously with 1.0  $\mu$ Ci (12  $\mu$ g) <sup>14</sup>C-vitamin D<sub>3</sub> and was killed 4 h after the injection by cervical dislocation. Pieces of the kidneys were quickly removed and frozen in isopentane cooled with liquid nitrogen. 5  $\mu$ m thick sections of the kidneys were then taken in cryostat ( $-15^\circ\text{C}$ ) in dark room and placed directly on photographic plates (K2 and G5, Ilford). The exposure was carried out at  $-15^\circ\text{C}$  for about 1 year. After the exposure the freeze-sections (remaining on the plates) were fixed in formalin. The plates were developed for 3 min in Kodak D19 and fixed, and the sections were stained with haematoxylin-eosin and mounted under cover glass, still on the plates. As controls several kidney-sections from untreated mice were taken and handled for autoradiography as described above.

### *Column chromatography*

Mice fed the vitamin D-deficient diet were injected intravenously with vitamin D<sub>3</sub> dissolved in 25  $\mu$ l absolute ethanol. One mouse received 0.1  $\mu$ Ci (4.8  $\mu$ g) H-vitamin D<sub>3</sub> and one mouse received 0.1  $\mu$ Ci H-vitamin D<sub>3</sub> together with 4.8  $\mu$ g non-labelled vitamin D<sub>3</sub>. 24 h later the animals were anesthetized with ether. The chest and the abdomen were opened, the vena cava was cut in front of the liver and the animals were perfused in the left ventricle with physiological saline, making the tissues almost empty of blood. The kidneys were removed, homogenized in 15 ml chloroform-methanol 2:1 (v/v) in Potter Elvehjem glass homogenizer and extracted by the method of Bligh and Dyer (1959). Thus the homogenate was shaken overnight at 4  $^\circ\text{C}$ . The chloro-

as was then separated after adding 2 volumes of ether. The aqueous methanol residue was further washed with 3 volumes of chloroform. The combined chloroform-phases were evaporated to dryness in vacuo and immediately redissolved in 0.2 ml *n*-hexane-chloroform 35:65 (v/v) to be used for chromatography. The column chromatography was performed at 4°C with Sephadex LH-20 eluted from Pharmacia Fine Chemicals, Uppsala, Sweden (Holsch and DeLuca 1971) using a column of 100 µm diameter with *n*-hexane-chloroform 35:65 (v/v). The same solvent system was used for elution of fractions and collected. The fractions were evaporated *in vacuo* to dryness in liquid scintillation and counted in a Packard liquid scintillation spectrometer after the addition of scintillation fluid (0.425 g POPOP per ml toluene). Correction for quenching was made by the use of an external standard. <sup>3</sup>H-vitamin D<sub>3</sub>, <sup>14</sup>C-vitamin D<sub>3</sub> and H-25-hydroxyvitamin D<sub>3</sub> were used as references to determine elution volumes.

#### Experiment 1

Measurement of radioactivity in the kidney after the injection of <sup>3</sup>H-vitamin D<sub>3</sub> as determined by mice fed the complete diet. Each mouse in the first series received 0.1 µCi (4.8 ng = 1.22 · 10<sup>-4</sup> H-vitamin D<sub>3</sub> dissolved in 25 µl abs. ethanol), the other series 0.1 µCi H-vitamin D<sub>3</sub> as mixed H-labelled vitamin D<sub>3</sub> and unlabelled (dissolved in 25 µl ethanol). Thus in the second series each mouse received 48 ng 1.22 · 10<sup>-4</sup> nmol vitamin D<sub>3</sub>, in the third series 0.48 µg = 1.22 nmol vitamin D<sub>3</sub>, in the fourth series 4.8 µg = 1.22 · 10<sup>-2</sup> nmol vitamin D<sub>3</sub> and in the fifth series 48 µg = 1.22 · 10<sup>-1</sup> nmol vitamin D<sub>3</sub>. Mice in each series were killed respectively 1 h, 2 h, 4 h, 8 h, 24 h and 48 h after the injection of vitamin D<sub>3</sub>. They were perfused as described above (see under Column chromatography) and their kidneys removed. These were then dissolved in toluene and the radioactivity as determined by scintillation counting. With the knowledge of the specific activity of the parent vitamin the disintegrations per minute in the samples were converted to the absolute amount (pmol) of steroid in the kidney.

#### Experiment 2

10 mice (fed the vitamin D-deficient diet) were injected i.c. with 0.1 µCi (4.8 ng) H-vitamin D<sub>3</sub> (dissolved in 25 µl dimethylsulfoxide). Six mice were killed at 4 h and six at 8 days after having first been on the deficient diet. Mice were injected in the same way with 0.1 µCi <sup>3</sup>H-vitamin D<sub>3</sub> together with 4.8 µg unlabelled vitamin D<sub>3</sub> and were killed at the same survival intervals as above (6 mice at each interval) after 1 hour perfused. The kidneys were removed and manually homogenized in 10 ml 0.25 M sucrose in a Beckman glass tissue grinder with teflon pestle (Kontes Ltd. N.J. USA). The tissue suspensions were filtered through a double layer of gauze. Cellular fractionation was performed by centrifugation in 0.25 M sucrose in a M.S.E. high speed centrifuge (M.S.E. Ltd., London, England) first at 650 g for 10 min to obtain the nuclear fraction, then at 16 000 g for 20 min to obtain the mitochondrial fraction and finally 50 000 g for 1 h to obtain the microsomal and supernatant fractions (see 109.7 mm). Microscopic examination of the nuclear fraction showed that it was practically free of contamination with unbroken cells (see below). The radioactivity of the fractions was determined by liquid scintillation counting after addition to toluene as described above.

In an additional experiment 10 mice were injected i.c. with 0.1 µCi <sup>3</sup>H-vitamin D<sub>3</sub> together with 4.8 µg unlabelled vitamin D<sub>3</sub> and were killed after 24 h. The kidneys were removed after perfusion and the nuclear and microsomal fractions were isolated as described above. These fractions were then diluted in 5 ml sucrose and subjected to sonication in a Branson Sonifier at 5A for 4–2 min (interruptions to cooling) in an ice bath. The sonified fractions were then centrifuged at 105 000 g for 1 h and the radioactivity in the resulting pellets and supernatants were determined by liquid scintillation counting as described above.

## Results

### Whole body autoradiography

After the injection of <sup>14</sup>C-vitamin D<sub>3</sub> there was no difference in the distribution of radioactivity between the mice fed the complete diet and those which received the vitamin deficient diet. At the studied survival intervals there was nor an obvious difference between the i.c. and the s.c. injected animals. In the description below no distinction will therefore be made between the different groups of animals.

Liver

a

Kidney

a

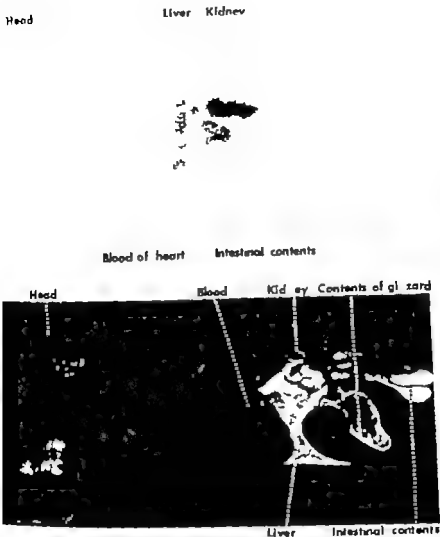
b

Liver

Kidney

Fig. 1 Autoradiographic distribution of vitamin D in the liver and the kidney. Details of whole autoradiograms of mice injected subcutaneously with 1  $\mu$ Ci (12  $\mu$ g)  $^3$ H-vitamin D<sub>3</sub> a) 1 h and b) 24 h prior to death. In a) high accumulation of radioactivity (white areas) is present in the liver. In the kidney the concentration of radioactivity is rather low. The cortex of the kidney shows a slightly higher concentration than the rest of the kidney. In b) the concentration of radioactivity in the liver is low. In the kidney a considerable amount of radioactivity is present in the labyrinthic part of the cortex.

At short survival intervals (5 min–4 h) after the administration of labelled vitamin D the radioactivity in the kidney was low in relation to organs such as the liver which accumulated a great amount of radioactivity. At eight hours a specific distribution pattern appeared in the renal cortex, but the radioactivity in the kidney was still lower than in the liver (Fig. 1 a). 24 h after the injection of labelled vitamin D the radioactivity in the renal cortex was high, exceeding that of all other tissues in the body except the gastrointestinal contents which contained a comparable amount of radioactivity (Fig. 2 a). At later survival intervals the radioactivity in the kidney dominated the distribution pictures. Eighteen days after injection a high concentration of radioactivity was still present in the renal cortex (Fig. 1 b).



2. Autoradiographic distribution of vitamin D<sub>3</sub>. Comparison between mouse and quail. Whole body radiograms of a) mouse and b) quail 24 h after subcutaneous injection of 0.5  $\mu$ Ci (4  $\mu$ g) respectively <sup>14</sup>C-vitamin D<sub>3</sub>. In the mouse, high concentration of radioactivity (black areas) is present in the intestinal contents—a comparable concentration of radioactivity is found only in the intestinal contents. The concentration of radioactivity in the liver is considerably lower than in the kidney. In the quail the concentration of radioactivity in the kidney is not exceeding the level of the blood. The concentration of radioactivity in the liver is considerably higher than in the kidney. The highest concentration of radioactivity is in the intestinal contents.

The radioactivity within the renal cortex was not evenly distributed but was concentrated in certain areas of the labyrinthic part. The extension of these areas indicated that they corresponded to the proximal tubuli. The remaining parts of the cortex, the medulla and the pelvis of the kidney had a low concentration of radioactivity. The same applied to the urinary bladder.



Fig. 3 Microautoradiographic distribution of  $^{14}\text{C}$  vitamin  $\text{D}_3$  in the kidney. Renal specimens from a mouse injected with  $3 \mu\text{Ci}$  ( $36 \mu\text{g}$ )  $^{14}\text{C}$ -vitamin  $\text{D}_3$  24 h prior to death were used for the autoradiography. Four sections were taken in a cryostat at  $-15^\circ\text{C}$  in a dark room directly on photographic plates. A glomerulus (G) with the connected proximal tubulus (P) is seen in the picture. Most silver grains are present in the proximal tubular cells. Hitz-eosun-G5 Ilford. ( $\times 300$ ).

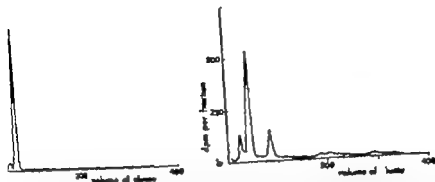
**Quails.** With respect to the kidney there was fundamental difference in the distribution patterns between quails and mice after the injection of  $^{14}\text{C}$  vitamin  $\text{D}_3$ . Thus in the quail there was no specific accumulation of radioactivity in the kidney at any of the studies survival intervals (Fig. 2 b). The concentration of radioactivity never exceeded that of the blood and there was no specific uptake in any part of the nephron. The highest concentration of radioactivity in the quails was observed in the liver and the intestinal contents.

#### Microautoradiography

The microautoradiograms showed the silver grains to be specifically located over the cells of the proximal convoluted tubuli (Fig. 3). The labelling was found to begin at the junction between the glomeruli and the tubuli. Specific labelling of sections from control mice was not observed.

#### Column chromatography

The profiles of extracts from the kidneys chromatographed on Sephadex LH 70 are shown in Fig. 4. When a high dose ( $4.8 \mu\text{g}$ ) of vitamin  $\text{D}_3$  was administered most of the radioactivity was chromatographed in a position corresponding to that of vitamin  $\text{D}_3$  (Fig. 4 a). In addition one radioactive peak was found which had a lower elution volume than vitamin  $\text{D}_3$ . This peak probably represents an ester of vitamin  $\text{D}_3$  (see Discussion). When a low dose ( $4.8 \text{ ng}$ ) of vitamin  $\text{D}_3$  was administered most of the radioactivity still was found to chromatograph in a position corresponding to that of vitamin  $\text{D}_3$  (Fig. 4 b). One radioactive peak had a position corresponding to 25-hydroxyvitamin  $\text{D}_3$ . As at the high dose one radioactive peak was also found which had a lower elution volume than vitamin  $\text{D}_3$ .



Radiochromatographic profiles of kidney extracts after the administration of  $^3\text{H}$ -vitamin D<sub>3</sub> to intact mice intravenously with  $^3\text{H}$ -vitamin D<sub>3</sub> 24 h prior to death, receiving 4.8  $\mu\text{g}$  (Fig. 4a) or 48  $\mu\text{g}$  (Fig. 4b). The homogenized kidneys were extracted with chloroform-methanol and the chloroform was chromatographed on 180 cm Sephadex LH20 column. N-hexane-chloroform 55:65 (v/v) as eluent solvent. 5 ml fractions were collected.

#### bioassay

As described above the autoradiograms showed that there was a slow uptake of radioactivity in the kidneys upon the injection of labelled vitamin D<sub>3</sub>. When this was studied by impulse counting (Table I) it was shown that there was an increase in the radioactivity in the kidney to a maximal level 24 h after the injection of vitamin D<sub>3</sub>. Still 48 h after the injection the radioactivity in the kidney was considerable, although lower than at the 24 h interval. It is interesting to note that, at dose levels ranging from 4.8 ng to 4.8  $\mu\text{g}$  of injected vitamin D<sub>3</sub>, the amount of steroid which was accumulated in the kidney was largely proportional to the given dose. At the highest dose level (48  $\mu\text{g}$ ) the accumulatory mechanism seemed to have been saturated, since the amount of steroid in the kidney was only about 2-4 times per dose the amount in the kidney at the lowest dose-level (4.8  $\mu\text{g}$ ).

#### cellular fractionation

The results of the cellular fractionation showed that most of the radioactivity in the kidneys after the administration of labelled vitamin D<sub>3</sub> was found in the mitochondrial and the

TABLE I. Amount of steroid in the kidney at different survival intervals after the injection of vitamin D<sub>3</sub> at different dose levels. Each mouse received intravenously 0.1  $\mu\text{Ci}$  (4.8  $\pm$  1.22  $10^{-6}$  nmol)  $^3\text{H}$ -vitamin D<sub>3</sub> only or together with non-labelled vitamin D<sub>3</sub> in doses of 48 ng (1.22  $10^{-6}$  nmol), 4.8  $\mu\text{g}$  (1.22  $10^{-5}$  nmol), 4.8  $\mu\text{g}$  (1.22  $10^{-5}$  nmol) and 48  $\mu\text{g}$  (1.22  $10^{-4}$  nmol). Three mice were used at each dose-level and survival interval. The radioactivity in their kidneys was determined. The disintegrations per minute in the samples were converted to the absolute amount (pmol) of steroid in the samples (mean  $\pm$  S.E.).

Amount of injected vit. D <sub>3</sub> (pmol)	Amount of steroid in the kidney per 100 mg kidney-tissue (pmol)					
	1 h	2 h	4 h	8 h	24 h	48 h
1.22 $10^{-6}$	6.1 $\pm$ 0.6 $10^{-6}$	6.4 $\pm$ 0.4 $10^{-6}$	7.2 $\pm$ 0.3 $10^{-6}$	12.0 $\pm$ 1.2 $10^{-6}$	17.3 $\pm$ 1.1 $10^{-6}$	13.0 $\pm$ 1.4 $10^{-6}$
1.22 $10^{-5}$	5.0 $\pm$ 0.8 $10^{-6}$	6.4 $\pm$ 0.7 $10^{-6}$	10.0 $\pm$ 0.9 $10^{-6}$	15.5 $\pm$ 1.0 $10^{-6}$	22.7 $\pm$ 1.5 $10^{-6}$	14.5 $\pm$ 0.5 $10^{-6}$
1.22 $10^{-4}$	4.0 $\pm$ 0.6	5.2 $\pm$ 1.0	6.1 $\pm$ 0.9	13.1 $\pm$ 9	28.6 $\pm$ 3.6	15.0 $\pm$ 1.2
1.22 $10^{-3}$	6.8 $\pm$ 0.9 $10^{-6}$	7.6 $\pm$ 0.7 $10^{-6}$	10.1 $\pm$ 1.4 $10^{-6}$	14.5 $\pm$ 0.6 $10^{-6}$	27.1 $\pm$ 2.3 $10^{-6}$	12.1 $\pm$ 0.1 $10^{-6}$
1.22 $10^{-2}$	4.2 $\pm$ 0.4 $10^{-6}$	—	4.1 $\pm$ 0.4 $10^{-6}$	5.6 $\pm$ 0.9 $10^{-6}$	5.9 $\pm$ 0.3 $10^{-6}$	3.4 $\pm$ 0.2 $10^{-6}$



Fig. 3 *Microautoradiograph distribution of vitamin D in the kidney* Renal specimens from a mouse injected with  $3 \mu\text{Ci}$  ( $36 \mu\text{g}$ )  $^3\text{H}$ -vitamin  $\text{D}_3$  24 h prior to death were used for the autoradiography. Five sections were taken in a cryostat at  $-15^\circ\text{C}$  in a dark room directly on photographic plates. A glomerulus (G) with the connected proximal tubulus (P) is seen in the picture. Most silver grains are present in the proximal tubular cells. Htx.-eosin, G5, Ilford. ( $\times 300$ ).

**Quails** With respect to the kidney there was fundamental difference in the distribution patterns between quails and mice after the injection of  $^3\text{H}$ -vitamin  $\text{D}_3$ . Thus in the quail there was no specific accumulation of radioactivity in the kidney at any of the studied survival intervals (Fig. 2 b). The concentration of radioactivity never exceeded that of the background and there was no specific uptake in any part of the nephron. The highest concentration of radioactivity in the quails was observed in the liver and the intestinal contents.

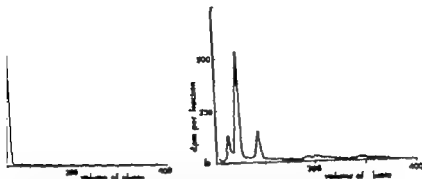
#### *Microautoradiography*

The microautoradiograms showed the silver grains to be specifically located over the apical surface of the proximal convoluted tubuli (Fig. 3). The labelling was found to begin at the junction between the glomeruli and the tubuli. Specific labelling of sections from control mice was not observed.

#### *Column chromatography*

The profiles of extracts from the kidneys chromatographed on Sephadex LH 20 are shown in Fig. 4. When a high dose ( $4.8 \mu\text{g}$ ) of vitamin  $\text{D}_3$  was administered most of the radioactivity was chromatographed in a position corresponding to that of vitamin  $\text{D}_3$  (Fig. 4 a). In addition one radioactive peak was found which had a lower elution volume than vitamin  $\text{D}_3$ . This peak probably represents an ester of vitamin  $\text{D}_3$  (see Discussion). When a low dose ( $4.8 \mu\text{g}$ ) of vitamin  $\text{D}_3$  was administered most of the radioactivity still was found to chromatograph in a position corresponding to that of vitamin  $\text{D}_3$  (Fig. 4 b). One radioactive peak had a position corresponding to 25-hydroxyvitamin  $\text{D}_3$ . As at the high dose one radioactive peak was also found which had a lower elution volume than vitamin  $\text{D}_3$ .





Radiochromatographic profiles of kidney extracts after the administration of  $^3\text{H}$ -vitamin D<sub>3</sub> to a special strain of mice with  $^3\text{H}$ -vitamin D<sub>3</sub> 24 h prior to death, receiving 4.8  $\mu\text{g}$  (Fig. 4) or 48  $\mu\text{g}$  (Fig. 5). The homogenized kidneys were extracted with chloroform-methanol and the chloroform as chromatographed on 1.80 cm Sephadex LH20 column, N-hexane-chloroform 35:65 (v/v) as eluent medium. 5 ml fractions were collected.

#### Counting

As noted above the autoradiograms showed that there was a slow uptake of radioactivity by kidneys upon the injection of labelled vitamin D<sub>3</sub>. When this was studied by impulse counting (Table I) it was shown that there was an increase in the radioactivity in the kidney to a maximal level 24 h after the injection of vitamin D<sub>3</sub>. Still 48 h after the injection the activity in the kidney was considerable, although lower than at the 24 h interval. Interesting to note that, at dose levels ranging from 4.8 ng to 4.8  $\mu\text{g}$  of injected vitamin D<sub>3</sub>, the amount of steroid which was accumulated in the kidney was largely proportional to the given dose. At the highest dose level (48  $\mu\text{g}$ ) the accumulatory mechanism seemed to be saturated, since the amount of steroid in the kidney was only about 2–4 times more than the amount in the kidney at the closest dose-level (4.8  $\mu\text{g}$ ).

#### Kidney fractionation

Results of the cellular fractionation showed that most of the radioactivity in the kidneys after the administration of labelled vitamin D<sub>3</sub> was found in the mitochondrial and the

TABLE I  
Amount of steroid in the kidney at different survival intervals after the injection of vitamin D<sub>3</sub> at different dose levels. Each mouse received intravenously 0.1  $\mu\text{Ci}$  (4.8  $\mu\text{g}$   $1.22 \cdot 10^{-4}$  nmol)  $^3\text{H}$ -vitamin D<sub>3</sub> only or together with non-labelled vitamin D<sub>3</sub> in doses of 4.8  $\mu\text{g}$  ( $1.22 \cdot 10^{-4}$  nmol), 48  $\mu\text{g}$  ( $1.22 \cdot 10^{-3}$  nmol), 4.8  $\mu\text{g}$  ( $1.22 \cdot 10^0$  nmol) and 48  $\mu\text{g}$  ( $1.22 \cdot 10^2$  nmol). Three mice were used at each dose-level and survival interval. The radioactivity in their kidneys was determined. The disintegrations per minute in the samples were converted to the absolute amount (pmol) of steroid in the samples (mean  $\pm$  S.E.).

Dose of D <sub>3</sub> mg	Amount of steroid in the kidneys per 100 mg kidney-tissue (pmol)					
	1 h	2 h	4 h	24 h	48 h	
$1 \cdot 10^{-4}$	$6.1 \pm 0.6 \cdot 10^{-4}$	$6.4 \pm 0.4 \cdot 10^{-4}$	$7.2 \pm 0.3 \cdot 10^{-4}$	$12.0 \pm 1.2 \cdot 10^{-4}$	$17.3 \pm 1.1 \cdot 10^{-4}$	$13.0 \pm 1.4 \cdot 10^{-4}$
$1 \cdot 10^{-3}$	$5.8 \pm 0.8 \cdot 10^{-4}$	$6.4 \pm 0.7 \cdot 10^{-4}$	$10.0 \pm 0.9 \cdot 10^{-4}$	$15.5 \pm 1.0 \cdot 10^{-4}$	$22.7 \pm 1.5 \cdot 10^{-4}$	$14.5 \pm 0.5 \cdot 10^{-4}$
2	$4.0 \pm 0.6$	$5.2 \pm 1.0$	$6.1 \pm 0.5$	$13.1 \pm 2.9$	$28.6 \pm 3.6$	$15.0 \pm 1.2$
$2 \cdot 10^0$	$6.3 \pm 0.9 \cdot 10^0$	$9.6 \pm 0.7 \cdot 10^0$	$10.1 \pm 1.4 \cdot 10^0$	$14.5 \pm 0.6 \cdot 10^0$	$27.1 \pm 2.3 \cdot 10^0$	$12.1 \pm 0.1 \cdot 10^0$
$2 \cdot 10^2$	$4.2 \pm 0.4 \cdot 10^2$	—	$4.1 \pm 0.4 \cdot 10^2$	$5.6 \pm 0.9 \cdot 10^2$	$5.9 \pm 0.3 \cdot 10^2$	$3.4 \pm 0.2 \cdot 10^2$

TABLE II Subcellular localisation of radioactivity in the kidneys after the injection of labelled  $D_3$ . Mice were injected s.c. with high (4.8  $\mu$ g) and low (4.8 ng) doses of  $^3H$ -vitamin  $D_3$  and after 24 hours and 8 days. Cellular fractionation was performed by centrifugation in sucrose. The radioactivity of the fractions was determined by liquid scintillation. The table shows the percent radioactivity in the respective fraction (mean  $\pm$  S.E.).

	High dose (4.8 $\mu$ g)		Low dose (4.8 ng)	
	4 h	8 days	24 h	8 days
Nuclei	13.2 $\pm$ 5.3	11.3 $\pm$ 4.9	12.9 $\pm$ 0.5	12.2 $\pm$ 2.2
Mitochondria	4.4 $\pm$ 9.6	45.6 $\pm$ 4.4	32.7 $\pm$ 1.6	36.9 $\pm$ 4.4
Microsomes	34.3 $\pm$ 4.5	27.8 $\pm$ 2.2	23.5 $\pm$ 0.8	39.7 $\pm$ 5.7
Supernatant	10.2 $\pm$ 0.3	15.3 $\pm$ 0.7	11.1 $\pm$ 0.2	11.0 $\pm$ 1.6

microsomal fractions (Table II). Upon sonication of these fractions most radioactivity was found in the 105 000  $g$ -pellet (97.4% of the radioactivity in the mitochondrial fraction and 90.4% of the radioactivity in the microsomal fraction (mean of two experiments)). The radioactivity seems to be tightly bound to the mitochondrial and microsomal membranes.

### Discussion

The present application of whole body autoradiography to study the distribution of  $D_3$  has demonstrated the unique property of the proximal tubuli of the mouse kidney to accumulate vitamin  $D_3$ . Eighteen days after the injection of labelled vitamin  $D_3$  a considerable amount of radioactivity was still present in the kidney. It is interesting to note that the accumulation of vitamin  $D_3$  in the kidney takes place both in the mice receiving the  $D_3$ -deficient diet and in those receiving the normal diet. Vitamin  $D_3$  has been reported not being excreted in the urine (Cruckshank *et al.* 1954). In our experiments a very small amount of radioactivity was found in the urine. In contrast to the situation in the mouse, the kidney was found to lack the ability to accumulate radioactivity above the level of the rest of the body after the administration of labelled vitamin  $D_3$ . A greater radioactivity has been reported to occur in the kidney after the administration of labelled vitamin  $D_3$  in rats than in mice (Lawson *et al.* 1971). Thus birds may lack a specific accumulatory mechanism for vitamin  $D_3$  present in the renal proximal tubuli of mammals. These differences may reflect important dissimilarities in the metabolism and/or physiological action of the vitamin, which should be noted.

There was a slow increase in the amount of steroid in the kidney upon the intraperitoneal injections of vitamin  $D_3$ , with a maximum being reached at 24 h. Most of the vitamin was present in a nonmetabolized form. Similar results have been reported in the rat (Norman and DeLuca 1963; Norman *et al.* 1964; Lawson *et al.* 1971). The reason for the slow uptake of vitamin  $D_3$  in the kidney is not clear. Metabolism of vitamin  $D_3$  prior to its accumulation in the kidney obviously does not explain the delayed uptake. The vitamin may enter the proximal tubule cells either via the tubular lumen, after having first been ultrafiltrated in the glomerulus, or direct from the circulation, in the latter case by an accumulative process that is slow. Vitamin  $D_3$  circulates in the plasma mainly attached to a transport protein (Thomson *et al.* 1959; Rikkers and DeLuca 1967; Peterson 1971; Edelstein *et al.* 1973). Perhaps

has to occur before the accumulation in the kidney takes place. Far more experiment to clarify these points.

In the kidney the proximal tubuli were found to accumulate the radioactivity in spite of a high dose of labelled vitamin D. This has also been shown to result in an accumulation of radioactivity in the proximal tubuli (Kodicek *et al.* 1961). The mitochondria were found to be the organelles which accumulated most of the radioactivity. Sonication of these organelles did not release the radioactivity. A similar result has been reported in the rat (Norman and DeLuca 1964). These structures thus have a large capacity for accumulating vitamin D<sub>3</sub>, the amount being largely proportional to the injected dose at several dose levels, as shown in the present study.

Vitamin D<sub>3</sub> was present in the kidney mostly in a non-metabolized form after administration of both high and low doses of the vitamin, it was obvious that a change in dose so changed the chromatographic profile from the Sephadex column—so that vitamin D<sub>3</sub> was found in the kidney when a low dose of vitamin D had been administered. In the rat the liver seems to be the major site of 25-hydroxylation of vitamin D (Norman and DeLuca 1969). *In vitro* formation of 25-hydroxyvitamin D in the rat kidney has been reported as not occurring (Horsberg 1970). The small peak which had a lower retention time than the vitamin D<sub>3</sub> probably represents an ester form of the vitamin. Vitamins have been found in the kidney and other tissues upon the administration of vitamin D<sub>3</sub> (Kodicek 1968).

The kidney is considered a site of action for vitamin D<sub>3</sub>, the parent vitamin as well as its metabolites are active in enhancing phosphate, sodium and calcium reabsorption (Gekle 1971 Puschett *et al.* 1972 a, b). These processes take place principally in the proximal tubules of the kidney (Durant and Watson 1967 Puschett *et al.* 1970), i.e. in the same areas where accumulation of radioactivity occurs after the administration of labelled vitamin D. Administration of high doses of vitamin D causes nephrocalcinosis most marked in the proximal tubuli (e.g. Giacomelli *et al.* 1964). It is also interesting to note that vitamin D deficiency in the rat is associated with proximal renal tubular acidosis (Gulgnard and Durand 1973) and an increased secretion of amino acids via the proximal renal tubuli (Kodicek 1971). In the rat vitamin D-deficiency has been reported to result in swollen and biologically damaged kidney mitochondria (DeLuca *et al.* 1960). Vitamin D has been reported to stimulate the release of calcium from isolated kidney mitochondria (DeLuca *et al.* 1967).

### References

- BR, E. G. and W. J. DYER, A rapid method of total lipid extraction and purification. *Canad. J. Biochem.* 1959 37 911-917.
- BRON, L., *Renale Aspekt der Rachitis. Nephrologisches Archiv* 1971 44 213-218.
- ROEDER, E. M., E. KODICEK and P. ARMSTRONG, The vitamin D content of tissues of rats given ergocalciferol. *Biochem. J.* 1954 58 173-175.
- DELUCA, H. P., B. REIMER, H. EISENBOCK and F. KASPER, Vitamin D and the structure of kidney mitochondria. *Biochem. Biophys. Acta (Amst.)* 1960 40 526-530.
- DELUCA, H. P., G. W. ENOBYRON and H. KASPER, The action of vitamin D and parathyroid hormone on calcium uptake and release by kidney mitochondria. *Proc. nat. Acad. Sci. (Wash.)* 1962 48 1484-1489.

- DENCKER, L. and H. TÄLVE, Distribution of vitamin D<sub>3</sub>: evidence of accumulation in renal proximal tubuli and thyroid parafollicular cells. *Experientia* (Basel) 1973 29 719-722.
- DUARTE, C. G. and J. P. WATSON, Calcium reabsorption in proximal tubuli of the dog nephron. *Amer Physiol* 1967 212, 1335-1360.
- EDELSTEIN, S., D. E. M. LAWSON and E. KODICEK, The transporting proteins of cholecalciferol and hydroxycholecalciferol in serum of chicks and other species. *Biochem. J* 1973, 133 417-426.
- FRASER, D. R. and E. KODICEK, Enzyme studies on the esterification of vitamin D in rat tissues. *Biochim* 1968 109 457-467.
- GERKE, D. J. STRÖMBER and D. ROSTOCK, The effect of vitamin D on renal inorganic phosphate reabsorption of normal rats, parathyroidectomized rats and rats with rickets. *Prober Res* 1971 3 40-52.
- GIACOMELLI, F., D. SPIRO and J. A. WIENER, Study of metastatic renal calcification at the cellular level. *J cell Biol* 1964 22 189-206.
- GUKONARD, J. P. and A. TORRADO, Proximal tubular renal acidosis in vitamin D deficiency rickets. *Acta paediatr* (Uppsala) 1973 62, 343-346.
- HOLICK, M. F. and H. F. DeLUCA, A new chromatographic system for vitamin D and its metabolites: resolution of a new vitamin D metabolite. *J Lipid Res* 1971 12, 460-465.
- HORSTING, M. and H. F. DeLUCA, In vitro production of 25-hydroxycholecalciferol. *Biochem Biophys Res Commun* 1969 36 251-256.
- HORSTING, M., *The enzymic conversion of cholecalciferol to 25-hydroxycholecalciferol*. Ph.D. Thesis, University of Wisconsin Madison, 1970.
- KODICEK, E., E. M. DARMADY and P. STRANACK, The localization of <sup>14</sup>C-labelled vitamin D<sub>3</sub> in the kidney. *Clin. Sci* 1961 20 185-195.
- KODICEK, E., The story of vitamin D from vitamin to hormone. *Lancet* 1974, 1 325-329.
- LAWSON, E. E. M., D. PELC, P. A. BILL, P. W. WILSON and E. KODICEK, Synthesis of (1,2-<sup>3</sup>H)-cholecalciferol and metabolism of (4-<sup>14</sup>C, 1,2-<sup>3</sup>H)- and (4-<sup>14</sup>C, 1-<sup>3</sup>H)-cholecalciferol in rachitic rats and chicks. *Biochem. J* 1971 121 673-682.
- NORMAN, A. W. and H. F. DeLUCA, The preparation of <sup>14</sup>C vitamins D<sub>2</sub> and D<sub>3</sub> and their localization in the rat. *Biochemistry* 1963 2 1160-1168.
- NORMAN, A. W. and H. F. DeLUCA, The subcellular location of <sup>14</sup>C-vitamin D<sub>3</sub> in kidney and intestine. *Arch Biochem. Biophys* 1964 107 69-77.
- NORMAN, A. W., J. LUND and H. F. DeLUCA, Biologically active forms of vitamin D<sub>3</sub> in kidney and intestine. *Arch. Biochem.* 1964 108 12-21.
- NUMEROI, P., H. L. SASSAMAN, A. RODGERS and A. E. SCHAEFER, The use of radioactive phosphorus in the assay of vitamin D. *J. Am.* 1955 33 13-21.
- PETERSON, P. A., Isolation and partial characterization of a human vitamin D-binding plasma protein. *J. Biol. Chem.* 1971 246 7748-7754.
- PUSCHETT, J. B., Z. AGUE, D. SENESEY and M. GOLDBERG, Micropuncture study of the relationship of vitamin D to phosphate transport. *Clin. Res* 1970, 18 700.
- PUSCHETT, J. B., J. MORANZ and W. S. KURNICK, Evidence for a direct action of cholecalciferol and 25-hydroxycholecalciferol on the renal transport of phosphate, sodium and calcium. *J. Clin. Invest* 1974, 53 373-383.
- PUSCHETT, J. B., P. C. FERNANDEZ, I. T. BOYLE, R. W. GRAY, J. L. OMDAHL and H. F. DeLUCA, The renal tubular effects of 1,25-dihydroxycholecalciferol. *Proc Soc. exp Biol (N.Y.)* 1972, 141 379-384.
- RIKKERS, H. and H. F. DeLUCA, An in vivo study of the carrier proteins of <sup>14</sup>C vitamins D<sub>2</sub> and D<sub>3</sub>. *Amer. J. Physiol* 1967 213 380-386.
- STENBOCK, H. and A. BLACK, Fat-soluble vitamin XVII: The induction of growth-promoting calcium properties in fats and their unsaponifiable constituents by exposure to light. *J. Biol. Chem.* 1934, 106 263-298.
- THOMAS, W. C., Jr., H. G. MORGAN, T. B. CONNOR, L. HADDOCK, C. E. BILLS and J. E. HOWARD, Studies of antirachitic activity in sera from patients with disorders of calcium metabolism and preliminary observations on the mode of transport of vitamin D in human serum. *J. Clin. Invest* 1959, 38, 1078-1085.
- ULLBERG, S., Studies on the distribution and fate of <sup>14</sup>C-labelled benzylpenicillin in the body. *Acta med. (Stockh.) Suppl.* 1954, 118 1-110.
- ULLBERG, S., Autoradiographic studies on the distribution of labelled drugs in the body. *Second U.N. Conf. Peaceful Use of Atomic Energy*, 1958 24 248-254.

# Gastric Acid Secretion in a Teleostean Fish: A Method for the Continuous Collection of Gastric Effluence from a Swimming Fish and Its Response to Histamine and Pentagastrin

By

BJÖRN HOLSTEDT

Received 6 May 1975

## Abstract

**Dr. B. Gastric acid secretion in a teleostean fish. A method for the continuous collection of gastric effluence from a swimming fish and its response to histamine and pentagastrin.** Acta physiol. scand. 1975. 95. 417-423.

and secretion has been measured in the codfish (*Gadus morhua*) equipped with catheter draining stomach, by measuring the water swallowed by the fish. Unstimulated acid secretion was found to be at most values not exceeding 8  $\mu\text{mol H}^+$ /kg/h. Intramuscular injection of histamine evokes a rapid secretion of gastric acid, with a maximum acid output of 300  $\mu\text{mol/kg h}$  occurring with 1 g histamine dihydrochloride. No acid response was obtained with pentagastrin.

**Key words:** Fish physiology, gastric acid secretion, histamine, pentagastrin

vertebrates possessing an acid secreting stomach that have been studied so far reveal that acid secretion can be stimulated by exogenous histamine, except in the rays (Babkin *et al.* 1968). Most of these studies have been carried out in the higher vertebrates, but few deals with the lower vertebrates, e.g. fishes.

In teleosts, gastric acid secretion has been studied by Smit (1967). Acid secretion was stimulated by distending the stomach with a piece of sponge, and the gastric juice was sampled by taking out the sponge and squeezing it in distilled water. Grgxyan *et al.* (1968), working with the European eel (*Salmon glanis*) showed that histamine elicited the secretion of an acid gastric juice. The method used for the collection of gastric secretions was a modified syringe-technique, with ligatures imposed upon the cardiac and pyloric parts of the stomach. The above workers employed sampling methods permitting only the collection of acid secreted in the stomach during a certain time. The present work describes a technique allowing the continuous collection of gastric effluent from unanaesthetized fishes and presents results showing that gastric acid secretion is dose-responsively stimulated by histamine, but is not affected by intramuscular administration of pentagastrin.

- DENCKER, L. and H. TJÄLVE, Distribution of vitamin D<sub>2</sub>: evidence of accumulation in renal tubuli and thyroid parafollicular cells. *Experientia* (Basel) 1973, 29: 719-722.
- DUARTE, C. G. and J. F. WATSON, Calcium reabsorption in proximal tubuli of the dog nephron. *Physiol.* 1967, 212: 1335-1360.
- EDELSTEIN, S., D. E. M. LAWSON and E. KODICEK, The transporting proteins of cholecalciferol hydroxycholecalciferol: serum of chicks and other species. *Biochem. J.* 1973, 135: 417-424.
- FRASER, D. R. and E. KODICEK, Enzyme studies on the esterification of vitamin D in rat tissues. *Am. J. Physiol.* 1968, 209: 457-467.
- GEKLE, D. J. STRÄDER and H. ROSTOCK, The effect of vitamin D on renal inorganic phosphate reabsorption of normal rats, parathyroidectomized rats and rats with rickets. *Prober. Res.* 1971, 5: 40-52.
- GIACOMELLI, P., D. SPINO and J. A. WIDMER, Study of metastatic renal calcification at the cellular level. *J. cell Biol.* 1964, 22: 189-206.
- GUGNARD, J. P. and A. TORRADO, Proximal tubular renal acidosis in vitamin D deficiency rats. *paediatr.* (Uppsala) 1973, 62: 543-546.
- HOLICK, M. F. and H. F. DELUCA, A new chromatographic system for vitamin D<sub>2</sub> and its metabolites: resolution of a new vitamin D<sub>2</sub> metabolite. *J. Lipid Res.* 1971, 12: 460-465.
- HORSTING, M. and H. F. DELUCA, In vitro production of 25-hydroxycholecalciferol. *Biochem. Biophys. Res. Commun.* 1969, 36: 251-256.
- HORSTING, M., The enzymatic conversion of cholecalciferol to 25-hydroxycholecalciferol. Ph.D. University of Wisconsin, Madison, 1970.
- KODICEK, E., E. M. DARMADY and F. STRAHACK, The localization of <sup>14</sup>C-labelled vitamin D<sub>2</sub> in the rat. *Clin. Sci.* 1961, 30: 185-195.
- KODICEK, E., The story of vitamin D from vitamin to hormone. *Lancet* 1974, 1: 325-329.
- LAWSON, E. E. M., B. PELC, P. A. BELL, P. W. WILSON and E. KODICEK, Synthesis of (1-<sup>3</sup>H)-cholecalciferol and metabolism of (4-<sup>14</sup>C, 1,2-<sup>3</sup>H<sub>2</sub>)- and (4-<sup>14</sup>C, 1-<sup>3</sup>H)-cholecalciferol in rachitic rats and chicks. *Biochem. J.* 1971, 121: 673-682.
- NORMAN, A. W. and H. F. DELUCA, The preparation of H<sup>14</sup>-vitamins D<sub>2</sub> and D<sub>3</sub> and their localization in the rat. *Biochemistry* 1963, 2: 1160-1169.
- NORMAN, A. W. and H. F. DELUCA, The subcellular location of H<sup>14</sup>-vitamin D<sub>2</sub> in kidney and liver. *Arch. Biochem. Biophys.* 1964, 107: 69-77.
- NORMAN, A. W., J. LUND and H. F. DELUCA, Biologically active forms of vitamin D<sub>2</sub> in kidney and liver. *Arch. Biochem.* 1964, 108: 12-21.
- NUMEROFF, P. H., L. SARRAMAN, A. RODOLAS and A. E. SCHAEFER, The use of radioactive phosphate in the assay of vitamin D. *J. N. I.* 1955, 55: 13-21.
- PETERSON, P. A., Isolation and partial characterization of a human vitamin D-binding plasma protein. *J. Biol. Chem.* 1971, 246: 7748-7754.
- PUSCHETT, J. B., Z. AGUIR, D. SCHENK and M. GOLDBERG, Micropuncture study of the relationship of vitamin D to phosphate transport. *Clin. Res.* 1970, 18: 700.
- PUSCHETT, J. B., J. MORANZ and W. B. KURNICK, Evidence for a direct action of cholecalciferol and hydroxycholecalciferol on the renal transport of phosphate, sodium and calcium. *J. clin. Invest.* 1971, 51: 373-385.
- PUSCHETT, J. B., P. C. FERNANDEZ, I. T. BOYLE, R. W. GRAY, J. L. ONDAHL and H. F. DELUCA, The renal tubular effects of 1,25-dihydroxycholecalciferol. *Proc. Soc. exp. Biol. (N.Y.)* 1972, 141: 379-382.
- RIKERS, H. and H. F. DELUCA, An in vivo study of the carrier proteins of H<sup>14</sup>-vitamins D<sub>2</sub> and D<sub>3</sub>. *J. Physiol.* 1967, 213: 380-386.
- STEENBOCK, H. and A. BLACK, Fat-soluble vitamin XVII: The induction of growth-promoting calciferol properties in fats and their unsaponifiable constituents by exposure to light. *J. Biol. Chem.* 1925, 263-298.
- THOMAS, W. C., Jr., H. G. MORGAN, T. B. CONNOR, L. HADDOCK, C. E. BILLS and J. E. HOWARD, Size of antitachy activity in sera from patients with disorders of calcium metabolism and preliminary observations on the mode of transport of vitamin D<sub>2</sub> in human serum. *J. clin. Invest.* 1959, 38: 1078-1084.
- ULLBERG, S., Studies on the distribution and fate of <sup>32</sup>P-labelled tetracycline in the body. *Acta med. (Stockh.) Suppl.* 1954, 118: 1-110.
- ULLBERG, S., Autoradiographic studies on the distribution of labelled drugs in the body. *Second U. N. Conf. Peaceful Uses of Atomic Energy* 1958, 24: 48-54.



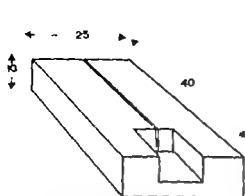


Fig. 1

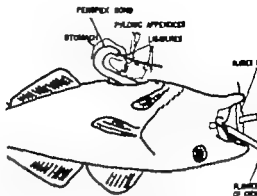


Fig. 2

Fig. 1. A clamp made from synthetic sponge was used to hold the fish during the operation. The fish was clamped in the longitudinal score (cut to 2/3 of the clamp's thickness) so that the head and gill-covers were placed in the frontal outcut. Measures in cm.

Fig. 2. Implantation of the catheter in the stomach of the anesthetized codfish.

### Material and Methods

**Animals.** Codfishes (*Gadus morhua*), weighing 290–810 g of both sexes were used. Before the experiment the fishes were kept in aquaria with circulating water at 10°C for at least one week. They were not fed.

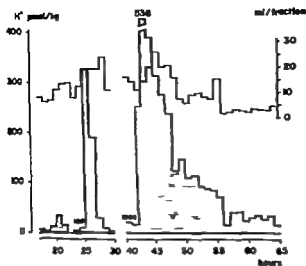
**Surgical procedure.** Anaesthesia was induced by immersing the fishes in water containing 0.01% Sandoz, and was complete when spontaneous ventilation ceased (2–5 min). The fishes were then taken to an operation table where respiration and anaesthesia were maintained by continuously flowing gills (about 1 l/min) with water M5-222. The fishes were placed ventral side up and firmly held in place by the clamp described below.

A convenient clamp for fish surgery was constructed from a piece of synthetic sponge rubber. The clamp should be longer than the fishes and is cut in the mid-line to 2/3 of its thickness. A slot of the sponge is cut out at one end to accommodate the head and allow the gill-covers to open. Care can be taken so that the gill-covers do not open in excess, so as to avoid only partial filling of the gills with water. By plugging in small pieces of sponges between the clamp and the gill-covers a suitable degree of the latter is easily achieved. Thus, the clamp can be adapted to accommodate fishes of different sizes. Between operations, the clamp is stored in a well aerated aquarium as this keeps it soft and at the same temperature.

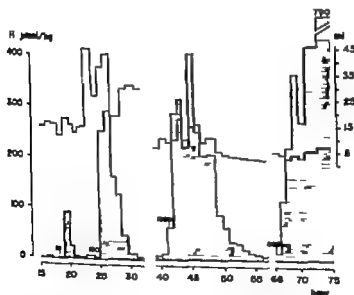
The abdominal cavity was opened by a midline incision, about 3 cm long, between the pelvic fins and anus. A perspex sond was then carried down the oesophagus into the stomach. One end of the sond was bent to form a half circle (diameter 30 mm), and was taken down the oesophagus with its bent end pointing towards the right side of the fish. Then the sond was rotated 90° around its long axis and manoeuvred so that the opening of the bent end apposed the pyloric sphincter. A catheter (from polyethylene tubing, PE-200) with a flanged back end, about 50 mm longer than the sond, was then inserted through the perspex sond. The PE-200 tube was cut flat at the leading end. The catheter was gently pushed through the pyloric valve and about 20 mm into the intestine where an incision in the intestinal wall was made, through which the tip of the catheter was taken out. 2 ligatures were then placed to prevent the catheter from slipping back (Fig. 2). By pulling the catheter through the sond the flange was placed close to the pyloric valve, it was prevented from sliding into the intestine by imposing a third ligature just proximal to the pyloric appendices. Care was taken not to interrupt the blood flow in the main vessels running across that area.

To remove the sond, it is pressed in the posterior direction and at the same time, manoeuvred so that its bent end is lowered (i.e. moved dorsally). During these movements, which involve no rotation around its long axis, the muscular pyloric part of the stomach is pinched between the thumb and index finger and pulled in the antero-ventral direction. The distensibility of the tissues now makes withdrawal of the sond out of the curved stomach possible. At this stage the bent end of the sond





A



B

secret of acid and water in two fishes following different doses ( $\mu\text{g/kg}$ ) of histamine dihydrochloride, at the time elapsed since operation.

### Discussion

Validity of the present method is based on the assumption that the water swallowed by fish is not regurgitated after having mixed with the stomach content, but is quantitatively released through the catheter. No critical test was performed to prove that this is the case. The arrangement of the catheter outlet secured an immediate delivery of the swallowed

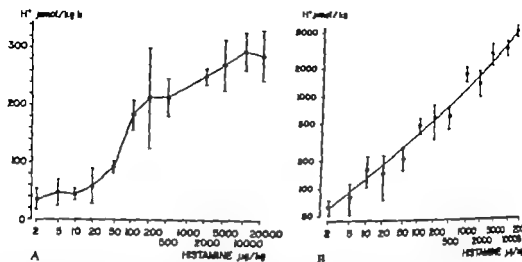


Fig. 3 Gastric acid secretion following graded doses of histamine dihydrochloride, given as rapid injections. Each fish was given 1-3 different doses. Values are means  $\pm$  S.E. of 3-8 determinations. Responses were calculated as the mean of the two hourly fractions containing most acid. The response following 1000  $\mu\text{g/kg}$  ( $362 \pm 53$ ,  $n=8$ ) has been excluded from the graph. *A* Responses are calculated the summed acid content of all fractions exceeding basal acid secretion.

dose-response curve, the values were calculated as the mean acid output of the two consecutive hourly fractions containing most acid. A delay in the appearance of this main peak with increasing doses of histamine was noticed: for doses up to 500  $\mu\text{g/kg}$  the peak appeared within 3 h following injection, up to 5 mg/kg the peak was attained 2-6 h following injection and for the highest doses as much as 10 h separated the injection from the peak output.

Fig. 4 shows examples of responses to different doses of histamine in two fishes. In addition to the increase of acid output there is also an increase in the duration of elevated acid production with increasing doses of histamine. Approximate values for the durations are given in Table I. The accumulated acid output, calculated as the sum of the acid content of fractions exceeding basal level, increased exponentially with increasing doses of histamine (Fig. 3 panel B).

In two cases, following 10 mg/kg histamine, blood appeared in the effluent water.

**Pentagastrin.** Pentagastrin was administered as rapid injections (25, 50, 100, 250,  $\mu\text{g/kg}$ ) and as slow injections during 5 h (50, 100, 200, 400  $\mu\text{g/kg h}$ ). In no case could stimulation of gastric acid secretion be detected.

Since pentagastrin is synthesized with mammalian gastrin as a model, species differences could explain the ineffectiveness of the pentapeptide. To demonstrate endogenous gastric codfish mucosae were extracted according to Blair *et al.* (1961) and the product tested for acid-stimulating power. No acid response has been obtained (preliminary results).

TABLE I. Duration of elevated acid secretion following i.m. injection of histamine dihydrochloride.

Dose range	Duration
2-50 $\mu\text{g/kg}$	3-4
100-200 $\mu\text{g/kg}$	4-6
500-1000 $\mu\text{g/kg}$	6-15
2-20 mg/kg	18-28

mean hourly output of acid following 5 mg/kg histamine amounts to 1 in the codfish.

It can be concluded to be a powerful secretagogue in the codfish. Whether it is instrumental in stimulating gastric acid secretion in the mammalia is a matter of controversy; the mechanisms in fish are totally unknown, elements enabling histamine to function as a final chemostimulator in the codfish seem to be fulfilled, histamine is present in the gastric secretions in considerable amounts (Reite 1969) and histamine powerfully stimulates gastric acid secretion (investigation).

It is suggested to exert its acid-stimulating effect via the release of histamine (Blom 1944), the secretion being sustained by an increased histamine release from the mucosa triggered off by a feed-back mechanism involving the mucosal histamine (Blom *et al.* 1964). According to a different opinion, the increased histamine release is mediated by gastrin (Johnson *et al.* 1969 Håkanson and Liedberg 1970).

Neither gastrin or pentagastrin has never before been tested in teleosts, like the codfish. No report showing the presence of gastrin in fish. As neither pentagastrin mucosal extract was effective in stimulating gastric acid secretion it is suggested that the gastrin mechanism does not exist in the codfish. If this is correct the codfish would be an interesting object for studying the correlations between gastric acid secretion and the activity of histidine decarboxylase.

Supported by grants from Adferbarta forskningsfonden and from Lars Hierta Minnefond for the animal material.

## References

- BLUM, H. F. and H. F. FRIEDMAN, Factors determining the course of gastric secretion. *Am. J. Med. Sci.* 1913. 1 251-259.
- A. HÄRTEL, H. J. LÄSSLE, J. D. REITZ and T. SCRATCHERD, A simple method of preparing histamine. (Lond.) 1961. 156. 11 P. 12 P.
- G. KAMLER, Histamine as a physiological stimulant of acid gastric secretion. *Acta physiol.* 1968. 2 288-304.
- M. G. ZARIN and O. F. TAMARITCHUK, Effect of histamine and pentagastrin on the gastric secretion of codfish (*Gadus morhua* L.). *Problems Ichthyol.* 1968. 2. 97-108. (Translation from 1968. 2. 126-130.)
- and G. LIDBERG, Effect of pentagastrin and feeding on gastric histamine decarboxylase activity in codfish. *Europ. J. Pharmacol.* 1972. 18. 31-36.
- J. ROSENBLUM, D. S. ARY and R. TITUSBERG, Mobilization and formation of histamine in the stomach as related to acid secretion. *J. Physiol. (Lond.)* 1964. 174. 400-416.
- V. Effect of food swallowing on the intensity of digestive processes in fishes. *Vopr. Zool.* 1977. 1-17.
- Stimulation by acid of gastric release. In: *Gastric*, ed. M. I. Grossman, UCLA Forum in Medicine. No. 5. University of California Press, 1966. 171-192.
- Effect of temperature on the rate of gastric juice secretion in the brown bullhead, *Ambystoma opacum*. *Biochem. Physiol.* 1967. 21. 125-132.

M. RUBIN, is known to the author only as cited by ZARIN H., Gastric secretion in lower vertebrates. In: *Handbook of physiology* Sect. 6 Vol. V. Ed. C. P. COOK. American Physiological Society, D.C. 1966. 2791-2805.

water quantitative recovery seems probable. The finding of above basal levels of acid in first obtainable fractions from fishes which did not drink during a 5-10 h period following operation also suggests that no leakage occurs through the oesophagus.

A comparison of volume and acid outputs (Fig. 4) reveals no correlation between the parameters. Often, an increase in acid output was accompanied by an increase in volume output or in some cases an increase in acid output was followed by an increased volume output. However, an increase in acid output may also occur simultaneously with a decreasing volume and *vice versa*. Furthermore, in the same fish, the coupling between acid and volume outputs is variable from one instant to another (*cf.* Fig. 4 panel B); following  $\mu\text{g/kg}$  histamine the increased acid output coincides with an increased volume output and the peak is delivered in a great volume of water. In contrast to this is the large amount of acid delivered in about one sixth of the volume following 10 mg/kg histamine. The independence between acid and volume outputs further suggests that the metric is reliable.

In most fishes, before drug injection, a slight acid production amounting to 1-5  $\mu\text{mol/l}$  could be detected. This basal acid secretion is 2-10 times as great as that reported by Grg *et al.* (1968) for the European catfish. Besides species differences, there is one methodological difference which might be relevant in explaining the discrepancy. In mammals the release of gastrin from the antral mucosa is suppressed by a low antral pH (see Schofield 1966). In fish such suppression is more likely to take place under conditions where the acid accumulates in the stomach (method of Grgzyan *et al.*) when the acid is continuously washed away (present method).

Krayukhin (1959), working with the bullhead (*Ictalurus nebulosus*) found that swallowing elicits an immense flow of gastric juice, and considers this to be evoked by an unconditioned reflex. Perhaps the basal acid secretion in the codfish could be explained by such unconditioned reflexes, as it can be assumed that in the current experimental conditions, the fish frequently swallow water. In contrast to the results obtained by Krayukhin (1959) on the bullhead the act of swallowing *per se* does not seem to be a secretory stimulus of importance in the codfish.

The acid concentration following histamine administration is not reported in the present investigation since this parameter is dependent on the volume of the water diluting the gastric juice. However, to facilitate comparison with data obtained by other workers on acid secretion in fish it might be pertinent to mention, that in low-volume samples (about 1 ml) acidities of 90 mM  $\text{H}^+$  have been encountered. After gastric distension the  $[\text{H}^+]$  for the bullhead was 249 mM at 25°C but at 10°C only 12 mM (Smit 1967). Grgzyan (1968) obtained from the catfish, after 500 mg/kg histamine, a gastric juice with an acidity of 0.3 g% (82 mM). The dose may be regarded as a maximal one since following 10 mg/kg histamine the catfish produced a juice of about 40 mM  $[\text{H}^+]$ .

The maximum rate of histamine induced gastric acid secretion in the codfish seems to be in the order of 300  $\mu\text{mol/kg h}$ , occurring with 10 mg/kg histamine. Again, this is less than the maximal rate of acid production in the bullhead (1060  $\mu\text{mol/h}$ , 25°C), but more than the bullhead's production at 10°C—25  $\mu\text{mol/h}$  (Smit 1967). Following 5 mg/kg histamine acid production in the catfish amounts to 4.5  $\mu\text{mol/h}$  (Grgzyan *et al.* 1968), but the

r 24 h. The mean hourly output of acid following 5 mg/kg histamine amounts to 00  $\mu$ mol/kg in the codfish.

may can thus be concluded to be a powerful secretagogue in the codfish. Whether histamine is instrumental in stimulating gastric acid secretion in the mammals under conditions is a matter of controversy the mechanisms in fish are totally different. Two requirements enabling histamine to function as a final chemostimulator of acid secretion in the codfish seem to be fulfilled, histamine is present in the gastric mucosa in considerable amounts (Reise 1969) and histamine powerfully stimulates gastric acid secretion (present investigation).

It has been suggested to exert its acid-stimulating effect in the release of histamine (de and Kahlson 1944), the secretion being sustained by an increased histamine capacity in the mucosa triggered off by a feed-back mechanism involving the mucosal histidine (Kahlson *et al.* 1964). According to a different opinion, the increased histidine activity is mediated by gastrin (Johnson *et al.* 1969 Håkanson and Liedberg

to my knowledge, gastrin or pentagastrin has never before been tested in teleosts, although there is any report showing the presence of gastrin in fish. As neither pentagastrin nor a codfish mucosal extract was effective in stimulating gastric acid secretion it is difficult to suggest that the gastrin mechanism does not exist in the codfish. If this is correct, codfish gastric mucosa would be an interesting object for studying the correlations between gastric acid secretion and the activity of histidine decarboxylase.

This study was supported by grants from Adherterska forskningsfonden and from Lars Hiertas Minne. I thank Mr. Ingemar Hakemar for the animal material.

## References

- BRADY, R. P. A. F. CHAMBERLAIN and H. F. FRIEDMAN, Factors determining the course of gastric secretion. *Annals of the New York Academy of Sciences* 1935. 1 251-259.
- DE MEYER, A. A. HARPER, H. J. LAKE, J. D. REID and T. SCRATCHERD, A simple method of preparing gastric juice. *J. Physiol. (Lond.)* 1961. 134. 11 P-12 P.
- REISE, W. and G. KAMMERS, Histamine as a physiological constant of acid gastric secretion. *Acta physiol. scand.* 1964. 2. 288-304.
- RYAN, D. M., M. G. ZAKS and G. F. TANABAYAN, Effect of histamine and plicamycin  $P^{10}$  on the gastric content of the European catfish (*Kribia glanis* L.). *Problems Ichthyol.* 1968. 2. 97-100. (Translation from Russian).
- LIEDBERG, E. and G. LARSSON, Effect of pentagastrin and feeding on gastric histidine decarboxylase activity in the teleost *Salmo gairdneri*. *Europ. J. Pharmacol.* 1972. 18. 31-34.
- REISE, G., E. RÖNNQVIST, D. SVANEN and R. TUNBERG, Mobilization and formation of histamine in the gastric mucosa as related to acid secretion. *J. Physiol. (Lond.)* 1964. 174. 400-416.
- JOHNSON, R. V. Effect of food swallowing on the intensity of digestive processes in fishes. *Vopr. Zool.* 1958. 17. 111-117.
- JOHNSON, R. V. Inhibition by acid of gastric release. In: *Gastric*, ed. M. I. Grossman. UCLA Forum in Medical Sciences. No. 5. University of California Press, 1966. 171-192.
- LI, H. Influence of temperature on the rate of gastric juice secretion in the brown bullhead, *Ambloplites nebulosus*. *Comp. Biochem. Physiol.* 1967. 21. 125-132.

The author, in Russia, is known to the author only as cited by SMYTH, H., Gastric secretion in lower vertebrates and birds. In: *Handbook of physiology* Sect. 6 Vol. V Ed. C. F. COOKE. American Physiological Society, Washington, D.C. 1963.

## On the Mechanism of Inhibitory Action of Vibrations as Studied in a Molluscan Catch Muscle and in Vertebrate Vascular Smooth Muscle

By

BENGT LJUNG and PER HALLGREN

Received 6 May 1975

---

### Abstract

---

LJUNG B. and PER HALLGREN *On the mechanism of inhibitory action of vibrations as studied in a molluscan catch muscle and in vertebrate vascular smooth muscle* Acta physiol. 1975. 95. 424-430

In previous studies longitudinal vibrations have been found to reduce active force development in muscle, possibly due to a direct action on the contractile mechanism. In the present experiments the inhibitory effect of vibrations on isometric tension was studied in isolated preparations of the rat portal and rabbit thoracic aorta and the anterior byssus retractor muscle (ABRM) in the *Mytilus edulis*. The experiments demonstrate that vibrations of appropriate frequency and amplitude caused prompt inhibition of contraction and that complete recovery of active force normally occurred after cessation of vibrations in vertebrate smooth muscle as well as during the phasic contraction of ABRM. However, in the "catch" ABRM there was no regain in force following the vibration induced inhibition. The contractile protein is considered to be in a locked state during the catch situation. Thus, this contracted state seems to be insensitive to vibrations. It is therefore concluded that vibrations do interfere with the interrelationship between myofibrils. This conclusion supports the previously forwarded hypothesis that vibrations act by increasing the rate of detachment of actin-myosin crosslinks in vertebrate smooth muscle.

---

Vibrational length changes have been found to reduce active isometric force even in vertebrate vascular smooth muscle (Ljung and Silvertsson 1972, 1975). Similar inhibitory effects of vibrations have previously been observed in striated muscle (e.g. Mathews 1966, Rack and Westbury 1969). There is indirect experimental support that this inhibition is caused by a mechanical interference with the contractile process in smooth muscle (Ljung and Silvertsson 1975) like in skeletal muscle, where an increased rate of actin-myosin crosslink detachment has been suggested to occur (Joyce *et al.* 1969).

In some molluscan muscles, such as the anterior byssus retractor muscle (ABRM) in *Mytilus edulis*, two different types of contractile responses can be elicited when either electrical means of stimulation are employed or environmental conditions are altered (see Low 1963, Millman 1963, Twarog 1967). The distinction is apparent from the rate of tension development after activation. Stimulation with alternating current, repetitive electrical impulses or

choline (ACh) at high temperatures produces a response which rapidly declines. Wherever current activation of ACh at "sea" temperature results in a contraction which only decays after stimulation. In the latter type of response two phases can be detected. The first component represents a tetanic contraction functionally similar to responses in brain smooth muscle. It is followed by a "catch" phase where the induced tension is maintained at a low metabolic rate (see Lowy and Millman 1963). Administration of 5-hydroxytryptamine (5-HT) to the muscle in catch phase leads to rapid relaxation. It is a matter of debate as to the exact mechanism by which the muscle maintains tension in the catch state (for see Rüegg 1971). However, there seems to be little doubt that the catch tension can be attributed to some process which prevents the sliding of adjacent contractile filaments relative to each other.

It appeared that a comparison of the influence of vibrations on active force in vertebrate skeletal muscle and on the phasic and catch components of lamellibranch smooth muscle responses would indicate whether vibrations inhibit active force by an action on the contractile mechanism. In the present experiments isolated preparations of the rat portal vein, rabbit thoracic aorta and the ABRM were exposed to longitudinal vibrations.

## Methods

Methods for studying the effects of vibrations on the vascular smooth muscle of the rat portal vein and a spiral strip of the rabbit thoracic aorta have recently been described in detail (Lyons and Sverrisdóttir 1972). In summary the vessels were carefully dissected after the animals had been sacrificed. The smooth muscle preparations, approximately 10 mm in length, were mounted in the longitudinal direction between two transducers (Grass FT 03) and a vibrator. The latter was fed sine wave current to impose length changes of  $\pm 400 \mu\text{m}$  in amplitude at a frequency of 100 Hz. A passive force of 5 and 15 mN was applied to the portal vein and the aortic spiral strip preparations, respectively.

The tissues were allowed to accommodate for one h before the experiment was started. The Krebs solution in which the vascular tissues had the following composition in mM: NaCl 122, KCl 4.73,  $\text{NaHCO}_3$  15.3,  $\text{CaCl}_2$  1.25,  $\text{CaCl}_2$  2.60 and Glucose 11.5. It was continuously bubbled with 4%  $\text{CO}_2$  in  $\text{O}_2$  and kept at  $37^\circ\text{C}$ .

In another experiment retractor muscle (ABRM) was obtained from fresh muscles (*Mus mus musculus*). The muscle was opened by cutting the adductor muscles. One of the retractor muscles was cut in order not to interfere with the other one. After removal of adjacent tissue the remaining retractor muscle was carefully split longitudinally into two halves. One half was firmly tied in situ with surgical silk to provide a 15 mm long section which was attached to the force transducer (Grass FT 03 with red & black springs) at one end and to the vibrator at the other end. The muscle was stretched to 70% of its *in situ* length in the presence of 10 $^{-6}$  M  $\text{BaCl}_2$ . The organ bath contained filtered seawater (690 mOsm), continuously bubbled with 4%  $\text{CO}_2$  in  $\text{O}_2$ . The temperature was normally kept at  $8-12^\circ\text{C}$ . The muscle was allowed to accommodate for one h before the actual experiment began.

The following drugs were used: 5-hydroxytryptamine (FLUKA AG), acetylcholine hydrochloride (E. Merck AG) and noradrenaline bitartrate (D-arterenol, SIGMA).

## Results

**Smooth muscle** Tracings from experiments on the rat portal vein preparation (A) and the rabbit thoracic aorta (B) are shown in Fig. 1. The spontaneous activity of the isolated portal vein preparation (A) consisted of phasic contractions occurring at a frequency of 2-4 per min with intervening periods of inactivity where the baseline level of passive force

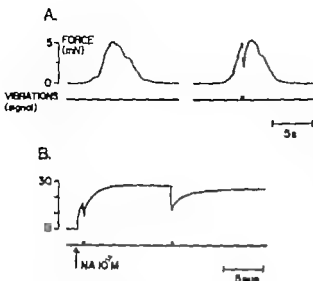


Fig. 1 Tracings of spontaneous contractions of the rat portal vein (A) and of a scorpion line (NA) induced response of the rabbit aorta (B). Vibrations applied at signal. The rapid decrease in force caused by vibration and regain in active force after the arrest periods.

prevailed. The left recording in Fig. 1 A represents one such spontaneous contraction. Mechanical vibrations (100 Hz,  $\pm 100 \mu\text{m}$ ) were applied during the period of time indicated by the signal in the right recording of panel A. It is seen that the vibrations caused a prompt decrease in active force, followed by a somewhat slower regain after the vibration period. Apart from this short lasting, vibration-induced interference with active force the configuration of the spontaneous contraction was not altered.

The NA response of the rabbit thoracic aorta (Fig. 1 B) was characterized by a smooth increase in force until a stable level of activity was reached. In the experiment illustrated in Fig. 1 vibrations were applied during the rising phase of the response to NA ( $10^{-6}$  M) and during the response plateau 10 min later. In both of these cases vibrations caused immediate reductions of active force of the aortic smooth muscle followed by a gradual recovery after termination of the oscillations. Thus, the imposed length changes induced reversible inhibitory responses in vascular smooth muscle. The rate of the recovery after cessation of vibration was greater in the portal vein than in the aortic preparation.

**Molluscan catch muscle** The contractile response of the ABRM to acetylcholine (ACh) at  $12^\circ\text{C}$ , is illustrated in the top recording of the left panel of Fig. 2. Typically a peak force level was reached during the first min after administration of ACh and thereafter force receded gradually. The relaxation occurred at the same low rate whether or not the agonist was rinsed out after 4 min exposure. Vibrations applied during the rising phase of the ACh response (middle and lower recording) caused a transient reduction of tension. In contrast, only little regain of force was seen when vibration-induced inhibition was elicited either 2.5 min after the administration of ACh, i.e. before rinsing, or after 5 min i.e. after ACh had been removed (middle and lower tracings, respectively of Fig. 2, left panel). The right panel of Fig. 2 illustrates responses to ACh of the ABRM in sea-water at  $37^\circ\text{C}$ . The control response (upper tracing) was characterized by a transient peak contraction which only displayed a phasic component. Vibrations applied 30 s after onset of the contraction resulted in an abrupt, short lasting inhibition. Occasionally a second phasic contraction was apparently elicited by vibrations (lower tracing). Thus, the imposed oscillations caused a



12°C

37°C

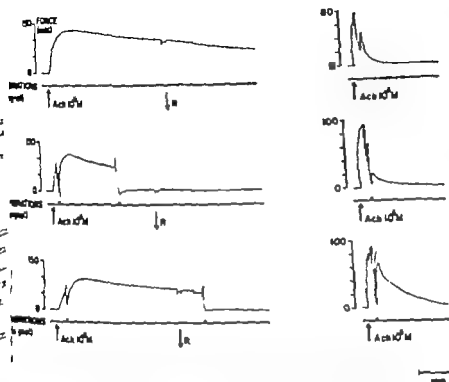


Fig. 2. Response of the ABRM to acetylcholine (ACh) in sea-water at 12°C (left panel) and at 37°C (right panel). Upper recordings show control responses to the agonist, which was rinsed out at R. Middle and lower recordings show effect of vibrations applied at signal. Note lack of force recovery after the second burst of vibration applied at 12°C (left panel, middle and lower recordings).

and pattern of reversible inhibition of the phasic response at 37°C as it did during the rising phase at 12°C. This is in contrast to the non-reversible inhibition of the ABRM obtained at 12°C 2.5 or 5 min after the induction of the response.

The tracings of Fig. 3 taken from one expt. on the ABRM with repeated exposures to ACh, illustrate the relationship between time of exposure to ACh and effects of vibrations. Brief oscillations applied after 5 and 10 min of ACh exposure (top and second tracings, respectively) caused complete and sustained abolition of active force. Subsequent applications of vibrations at decreasing time intervals after the ACh injection were followed by increasingly more pronounced recoveries, so that the contraction amplitude which ensued vibrations applied 15 s after ACh injection (bottom recording) was actually greater than the initial peak response level. The sequence of effects of vibrations in Fig. 3 thus demonstrates that the degree of maintained inhibition following the oscillations depends on the time elapsed after the administration of the agonist.

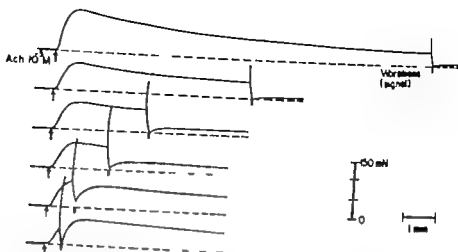


Fig. 3 Effect of vibrations on ABRM responses to acetylcholine (ACh). Vibrations applied at different periods after administration of the agonist. Note that increasingly greater recoveries occurred between onset of response and application of vibrations.

The same sequence of vibrations, applied during a single period of ACh exposure, is illustrated in Fig. 4. The control response to ACh is shown by the contour of the shaded area. After repeated rinses and complete muscle recovery, ACh was again administered and vibrations were applied after 0.3, 0.6, 1.25, 2.5, 5 and 10 min. It is seen from the two superimposed recordings that during the rising phase of the response, pronounced inhibition followed by complete recovery. From the peak response level and on, successless regain of force was seen after vibrations and 5 min after ACh injection hardly any tension was reestablished.

### Discussion

Vibrations were found to cause prompt reductions of active force when applied in the experiments. Complete regain of tension gradually followed the brief vibration ;

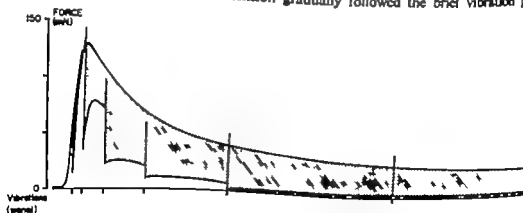


Fig. 4 Effects of vibrations on ABRM response to acetylcholine ( $10^{-5}$  M). Experiment recorded on and replayed on rectilinear recorder. The upper contour of the shaded area represents control response and that of the open area shows a superimposed recording of a subsequent response where vibrations were repeatedly applied in signal. Note that complete recovery of the contraction occurred after the usual delay not after subsequent vibrations.

vertebrate smooth muscle and under certain conditions in the ABRM. In contrast, any recovery is seen when vibrations were applied a few minutes after the onset of action in experiments at a temperature of 12°C. Thus, there is a clear distinction between the reversible inhibition found in vertebrate smooth muscle, in the ABRM at 37°C and the early response of the ABRM on the one hand and the maintained inhibition during the steps of the ABRM response at 12°C on the other hand.

Particular properties of some molluscan muscles, like the ABRM, have attracted interest. It is known that stimulation with either alternating current (Winton 1937) or nerve impulses (Lowy and Milfman 1963), or with exogenous ACh at a temperature of 37°C (T frog 1967) leads to a "phasic" contraction which quickly decays. If instead ABRM is exposed to direct current or ACh at low temperatures, the initial "phasic" one is followed by a maintained tension associated with low energy consumption and given away very slowly. Since the ABRM appears to be caught, this contraction phase is called the catch of the muscle. It seems clear that the maintained inhibition obtained in ABRM after vibration reflects release of the catch mechanism. Thus, the progressive increase of regained force found with increasing interval between onset of response and location of vibrations (Fig. 3) is explained by the time course of the phasic response potent. Repeated exposures to vibrations during one single response (Fig. 4) would separate the contribution to the force development provided by the active and the catch tension, respectively. Two main explanations have been given for the catch mechanism, leading to the paramyosin hypothesis: the sliding of adjacent contractile protein filaments is hindered by shearing forces applied by the paramyosin protein, which is abundantly present in catch muscles (see Ruegg 1971). The "linkage hypothesis" forwarded by Lowy, Leam and Hansson (1964) suggests that the crosslinks between actin and myosin filaments, cyclically formed and detached during the early phase of contraction, only detach very slowly during the catch phase.

The present experiments do not seem to offer conclusive support for either of the two functions of catch. However, they do demonstrate that vibrations abolish the catch tension. This effect must be accomplished by a mechanical separation of the caught contractile elements. It is reasonable to conclude that the reversible inhibition of the phasic ABRM contraction likewise is due to interference with the contractile process, i.e. detachment of arthroserous bindings.

By analogy, the present results strongly support the hypothesis (Ljung and Sjöström 1975) that the pronounced inhibitory effects of oscillating length changes in vertebrate smooth muscle is due to increased rate of detachment of crosslinks between the contractile filaments.

Supported by the Swedish Medical Research Council (3834). Thanks are due to Miss Ann Kjellstedt for technical assistance and to Miss Lena Nilsson for secretarial aid.

### References

- LEE, G. C., P. M. BLACK and D. R. WESTERMARK. The mechanical properties of cat soleus muscle during controlled lengthening and shortening movements. *J. Physiol. (Lond.)* 1969, 204: 441-474.  
 LOWY, R. and R. SEVATSKY. The inhibitory effect of vibrations on tension development in vascular smooth muscle. *Acta physiol. scand.* 1972, 85: 428-430.

- LJUNG, B. and R. SEVERTSON, Vibration-induced inhibition of vascular smooth muscle contraction. *Acta physiol. Scand.* 1975 **12**, 38-52.
- LOWY J and B. M. MILLMAN, The contractile mechanism of the anterior byssus retractor muscle of *Mytilus edulis*. *Philos. Trans.* 1963 **B**, 246, 105-148.
- LOWY J., B. M. MILLMAN and J. HANSON, Structure and function in smooth tonic muscles of lamellibranch molluscs. *Proc. roy. Soc. B* 1964 **160**, 525-536.
- MATTHEWS, P. B. C., The reflex excitation of the soleus muscle of the decerebrate cat caused by vibration to its tendon. *J. Physiol. (Lond.)* 1966, **184**, 450-472.
- RÜZÖD, J. R. Smooth muscle tone. *Physiol. Rev.* 1971 **51**, 201-248.
- TWAROG, B. M. Factors influencing contraction and catch in *Mytilus* smooth muscle. *J. Physiol. (Lond.)* 1967 **192**, 847-856.
- WINTON, P. R., The changes in viscosity of an unstriated muscle *Mytilus edulis* during and after stimulation with alternating, interrupted and uninterrupted direct currents. *J. Physiol. (Lond.)* 1937 **88**, 493.

## Rubrospinal Control of Static and Dynamic Fusimotor Neurons

By

B. APPELBERG, T. JENSSON and H. JOHANSSON

Received 7 May 1975

---

### Abstract

---

Umeå, B., T. JENSSON and H. JOHANSSON. *Rubrospinal control of static and dynamic fusimotor neurons*. Acta physiol. scand. 1975. 95. 431-440.

Equal effects on about 60 extracellularly recorded  $\gamma$ -motoneurons were studied in anesthetized cats. All were retrogradely identified from sensory muscle nerves.  $\Delta$  cells were regarded as dynamic as they were activated from a mesencephalic region previously known to influence selectively muscle spindle sensitivity. The pattern of rubrospinal influence on static fusimotor neurons to different muscles followed that previously demonstrated for  $\alpha$ -motoneurons with predominantly excitation of flexors and excitation or inhibition in equal amounts of extensor cells. Dynamic fusimotor neurons reflected in strictly reciprocal manner with excitation of flexor cells and inhibition of extensor cells except for a few neurons which could not be reached from nucleus ruber. Evidence was also obtained indicating that the shortest path from nucleus ruber to static fusimotor neurons involves one synapse.

---

The effect of electrical stimulation within the red nucleus on the fusimotor system was first described by Appelberg (1962) and by Appelberg and Kosary (1963) by the indirect method recording from muscle spindle afferents. It was regularly observed that such stimulation excited and inhibited of spontaneous activity in extensor spindles while flexor spindles were excited. These findings were in general agreement with the notion at that time that the red nucleus was excitatory to flexor and inhibitory to extensor muscles (Sasaki, Nambu and Hashimoto 1960).

In recent years the control function of the rubrospinal tract as well as of other systems descending from the mesencephalon and acting on the neuronal machinery in lumbar segments have been extensively and carefully studied by Lundberg and coworkers (*cf* Hongo, Nishikawa and Lundberg 1969 a, b 1972 a, b and Baldissera, Lundberg and Udo 1972 a, b). These studies have, among other things, given extremely detailed information about rubrospinal control of lumbar  $\alpha$ -motoneurons and also carefully analyzed methodological aspects of importance when using electrical stimulation in the rubral region.

With this in mind and considering current theories of linkage between  $\alpha$ - and  $\gamma$ -motoneurons in motor control it seemed of great interest to reinvestigate in greater detail and

- LJUNG, B. and R. SEVERTZSON, Vibration-induced inhibition of vascular smooth muscle contraction. *Circula.* 1975. *52*, 38-52.
- LOWY J and B. M. MILLMAN The contractile mechanism of the anterior byssus retractor muscle of *Mytilus*. *Philos. Trans.* 1963. B, *246*, 103-148.
- LOWY J., B. M. MILLMAN and J. HANSON Structure and function in smooth tonic muscles of lamellib mollusca. *Proc roy Soc B* 1964 *160* 525-536.
- MATTHEWS, P. B. C., The reflex excitation of the soleus muscle of the decerebrate cat caused by its tendon. *J Physiol. (Lond.)* 1966. *184*, 450-472.
- RÜGG, J. R., Smooth muscle tone. *Physiol. Rev.* 1971 *51* 201-248.
- TWAROG, B. M. Factors influencing contraction and catch in *Mytilus* smooth muscle. *J Physiol.* (L) 1967 *192*, 847-856.
- WYTON, F. R., The changes in viscosity of an unstriated muscle *Mytilus edulis* during and after stimulation with alternating, interrupted and uninterrupted direct currents. *J Physiol. (Lond.)* 1937 *82*, 49.

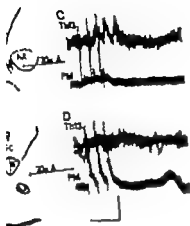


Fig. 1

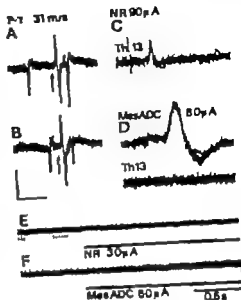


Fig. 2

1. A and B are camera lucida drawings of transverse sections through the mesencephalon at rostral (B about 1 mm caudal to A) with stimulating sites indicated. These stimulating shocks at 600 Hz are as A activates rubrospinal tract as revealed by surface recording from the cord in C (upper trace) as A activates rubrospinal tract as revealed by surface recording from the cord in C (upper trace). The stimulating point in B causes no rubrospinal volley (D, upper trace) but instead descending volley in the posterior lobe of the cerebellum is evoked (D, lower trace). Position is recorded as shown in this and all subsequent figures. Time calibration 5 ms, voltage calibration 100  $\mu$ V to upper trace in C and D, 200  $\mu$ V to lower traces.

2. A, B, microstimulation of plantar fusimotor neurons (see text). C, descending volley in Th13 to NR. D, PM-responses evoked from MesADC (upper trace). Note lack of descending volley (lower trace). Time calibration below B is 10 ms to A, B and D, 4 ms to C. Voltage calibration is 100  $\mu$ V for all. New trace at D E, inhibition of spontaneous fusimotor activity by repetitive stimulation in NR during NR is indicated by black line below record. F activation of same fusimotor neurons from MesADC.

An equally selective activation of the descending pathway to dynamic fusimotor neurons was attempted by placing second stimulating electrode dorsally in the low-threshold region for D-zone chloride responses in the PM. A direct activation of rubrospinal cells could usually be obtained from the PM, but only at high stimulating intensities. Only effects on  $\gamma$ -motoneurons at strengths well below threshold for NR-activation were considered. Also at such lower stimulating strengths descending volleys could sometimes be recorded in the thoracic segment during trans stimulation. This volley was similar to the one described by Ballieux *et al.* (1972) as being characteristic for the dorsal reticulo-spinal system. Fig. 1 the position of stimulating sites for rubro-spinal activation (A) and within the MesADC (B) is shown. In some experiments, here histological control was made, as illustrated. In C and D the physiological criteria for correct placement in all experiments are illustrated. In few early experiments only NR-stimulation took place and of the total cell-material of 63 cells 11 were thus not tested from MesADC (see next section).

## Results

### 1. Effects from NR and MesADC on $\gamma$ -motoneurons

Table 1 summarizes the results obtained in one experiment (same experiment as Fig. 1). NR-stimulation was applied from 6 of which were completely tested with regard to

with another technique the matter of rubrospinal control of hind limb fusimotor output. A detailed knowledge of a descending system selectively influencing dynamic fusimotor neurones (*cf.* Appelberg and Jeneskog 1972, Jeneskog 1974a) was utilized as a methodological tool allowing the separation of  $\gamma$ -motoneurones into the two functional groups, static and dynamic.

## Methods

The results to be presented were obtained in experiments on 18 cats. Of these 11 were anaesthetized with halothane (usually 1.0–1.5%) administered in a mixture of Oxygen (1/3) and Nitrous Oxide (2/3), 4 mg  $\alpha$ -Chloralose (60 mg/kg) administered intravenously after induction with halothane and 3 mg per barbital (40 mg/kg) given intraperitoneally with later supplementary intravenous doses. Essentially similar findings were obtained regardless of type of anaesthesia. Blood pressure and expiratory  $\text{CO}_2$ , as well as rectal temperature and temperatures in the paraffin pools were continuously monitored throughout the experiments.

## Operation

The operative procedure included a low thoracic and a lumbar laminectomy. The dorsal column and right spinal half were sectioned in T<sub>12</sub>–L<sub>3</sub>. The lumbar spinal cord was prepared for microelectrode recording from hind limb motoneurone pools.

In the left hind limb the following nerves were dissected and mounted for stimulation: posterior biceps semitendinosus (PSt), anterior biceps-semimembranosus (ABSt), gastrocnemius-soleus (GS), plantar (P), flexor digitorum and hallucis longus (FDL), deep peroneal (DP), superficial peroneal (SP) and tibial (Tib). In one experiment the tenuissimus nerve was prepared instead and later used for dissection of filaments (*see* Results, Fig. 3).

The sensorimotor cortex was acutely ablated bilaterally in all experiments.

Access to the right mesencephalon for stimulating electrodes and to the left paramedian lobule of the cerebellum for surface recording was arranged.

## Recording

Extracellular recording was made from  $\gamma$ -motoneurones with glass capillary electrodes filled with 4 M NaCl. The impedance of the electrodes was usually about 1–2 M $\Omega$ . Only cells which could be antidromically invaded from one of the dissected muscle nerves and thus identified were accepted. The conduction velocities of the axons could then be determined by also measuring the conduction distance along the dissected nerve after each experiment. Only cells having a spontaneous activity could be tested with regard to a facilitatory influence from the brain, and therefore each cell included in the material exhibited such an activity during at least part of the recording session.

Surface recording of the rubrospinal tract volley on the dorsolateral aspect of the cord in the exposed thoracic segment was employed to guide the placement of a stimulating electrode in the red nucleus (RN).

Surface recording of climbing fibre responses in the D-zone of the cerebellar paramedian lobule (PL) was employed when placing a stimulating electrode in a mesencephalic area previously shown to selectively influence dynamic fusimotor neurones (MesADC—*cf.* Appelberg 1967 and Jeneskog 1974a and Dönnestål *et al.* 1974).

## Stimulation

For stimulation in the brain two medio-lateral grids of 3 glasscovered platinum-mesh electrodes of 1 mm interelectrode distance were used. One of the grids was angled 15° to the critical plane as all electrodes had to be positioned at approximately the same rostro-caudal level but at different depths.

A presumably selective activation of the rubro-spinal tract was obtained by stimulation of interposed rubral fibres in the caudo-ventral part of or just ventrally to the caudal part of the red nucleus. The correct placement of the stimulating electrode was aided by observing the descending volley changes from a short latency direct response to a transsynaptic response with longer latency when slowly proceeding with the electrode through the red nucleus (*cf.* Baldessarini *et al.* 1972b). This placement allowed maximal activation of the rubro-spinal tract at low stimulating strength at a considerable distance away from the MesADC.



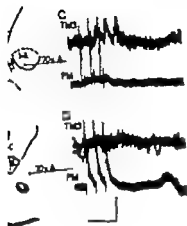


Fig. 1

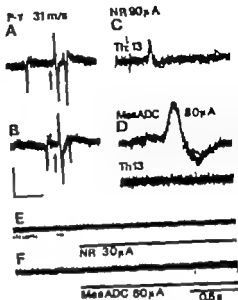


Fig. 2

A and B are camera lucida drawings of transverse sections through the mesencephalon at rostral (B about 1 mm caudal to A) with stimulating sites indicated. Three stimulating shocks at 600 Hz (as in A) activates rubrospinal tract as revealed by surface recording from the cord as C (upper trace). The stimulating point in B causes no rubrospinal volley (D, upper trace) but instead climbing response as the parabrachial lobes of the cerebellum is evoked (D, lower trace). Polarity is recorded as in this and all subsequent figures. Time calibration 5 ms, voltage calibration 100  $\mu$ V to upper trace in C and D, 200  $\mu$ V to lower traces.

2. A, B, identification of plantaris flexor motor neurone (time test). C, descending volley at Th<sub>13</sub> to NR. D, FM-response evoked from MesADC (upper trace). Note lack of descending volley (lower trace). Time calibration below B is 10 ms to A, B and 4 ms to C. Voltage calibration is 100  $\mu$ V for A, B and 200  $\mu$ V for C, D, E. Inhibition of spontaneous flexor motor activity by repetitive stimulation in NR during F indicated by black line below record. F activation of same flexor motor neurone from MesADC.

In equally selective activation of the descending pathway to dysogenic flexor motor neurones was attempted by placing second stimulating electrode dorsally in the low-threshold region for D-zone climbing response at the FM. A direct activation of rubrospinal cells could usually be obtained from that place, but only at high stimulating intensities. Only effects on  $\gamma$ -motoneurons at strengths well below the needed for NR-activation are considered. Also at such lower stimulating strengths the descending volley was recorded as the thoracic segment during train stimulation. This volley is similar to that described by Balchowitz *et al.* (1972a) as being characteristic for the dorsal reticulo-spinal system. 1. C and D show the position of stimulating areas for rubro-spinal activation (A) and when the MesADC (B) in one experiment, where histological control was needed, as illustrated. In C and D the physiological criteria used to select placement in all experiments are illustrated. In a few early experiments only NR-stimulation was placed and of the total cell-material of 63 cells 11 were thus not tested from MesADC (see under Results).

## Results

### 1. Effects from NR and MesADC on $\gamma$ -motoneurons

Table 1 summarizes the results obtained in one experiment (same experiment as Fig. 1). Nine  $\gamma$ -motoneurons were recorded from, 6 of which were completely tested with regard to

TABLE I Effects from nucleus ruber (NR) and mesencephalic area for dynamic control of muscle (MesADC) on nine fusimotor neurones in 1 expt. Thresholds for effects given in  $\mu\text{A}$ . + = excitation, - = inhibition 0 = no influence.

Cell no.	Type	Cond. vel.	NR	$\mu\text{A}$	MesADC	$\mu\text{A}$
1	FDL	32 m/s	-	50	+	60
2	DP	32 m/s	+	50	0	70
3	DP	27 m/s	+	60	+	70
4	DP	34 m/s	+	30	+	60
5	DP	27 m/s	+	40	+	70
6	DP	30 m/s	+	40	+	40
7	DP	23 m/s	+	70		
8	PBSI	16 m/s	+	60		
9	FDL		-		0	

Threshold for rubrospinal volley from NR 30  $\mu\text{A}$ .

Threshold for rubrospinal volley from MesADC 90  $\mu\text{A}$ .

Threshold for PM-response from MesADC 30  $\mu\text{A}$ .

effects evoked from the two central regions, while 3 cells were kept only for short periods and not fully tested. The threshold for evoking a rubrospinal volley from the electrode just ventrally to the NR was about 30  $\mu\text{A}$ . From the electrode placed in MesADC a rubrospinal volley could be evoked at stimulating strengths above 90  $\mu\text{A}$ . A PM-response was, on the other hand, evoked already at 30  $\mu\text{A}$ . It is clear from the Table that all of the different cells were obtained at stimulating intensities which makes spread of cells from one region to the other unlikely. This conclusion is supported by the fact that cell 1 which was reciprocally influenced from the two regions at low stimulating intensities, controlled in the same way but stronger when stimulation was increased to 90  $\mu\text{A}$  in NR and to 80  $\mu\text{A}$  in MesADC. The central effects on all cells included in the material presented have been studied under similar circumstances.

A cell influenced in the same reciprocal way as the FDL  $\gamma$  (cell 1) in Table I but belonging to the plantaris muscle is illustrated in Fig. 2. This cell was slightly difficult to identify and responded antidromically from the nerve together with another cell (A). From the records in A and B it appears, however, that when a spontaneous spike of the cell occurred close enough to the antidromic stimulus (B) a collision of orthodromic and antidromic spikes took place in the axon and the antidromic response disappeared. The remaining smaller spike belonging to the same nerve was not spontaneously active, nor was it influenced by the central stimuli. The rubrospinal volley evoked from the NR-electrode is illustrated in C, and the characteristic PM-response from the MesADC-electrode in D (note lack of volley in Th 13). In Fig. 2 E is seen a strong inhibitory influence on the spontaneously active cell from NR at low stimulus intensity. In F the cell is instead shown to be strongly activated from MesADC. In this experiment the thresholds for detectable descending rubrospinal volley from the NR-electrode and for PM response from the MesADC-electrode were both around 40  $\mu\text{A}$ . A weak rubrospinal volley for MesADC-stimulation was seen at 100  $\mu\text{A}$ .

In Table II the whole material of 63 cells and the effects from MesADC and NR is presented. The cells have been divided into 3 subpopulations on the basis of the effects upon them from MesADC. As previous work on muscle spindle afferents has revealed that the

Effects from NR and MesADC on whole material of 63 cells.

not influenced from MesADC (Static cells)								Extensor	Flexor
from NR	GS	FDL	P	ARden	PRSt	DP			
m	3	0	0	1	9	3		6	14
u	3	1	1	0	0	0		7	0
r	10	1	1	1	9	3		13	14
influenced from MesADC (Dynamic cells)								Extensor	Flexor
from NR	GS	FDL	P	ARden	PRSt	DP			
m	1	0	0	3	4			1	0
u	3	3	2	0	0			6	0
r	4	0	0	0	1			4	1
r	8	3	2	3	5			13	10
Is not tested from MesADC								Extensor	Flexor
from NR	FDL	P	ARden	Tib	PRSt	DP	Ten		
m	1	1	1	1	3	4	1	4	8
u	0	0	0	0	0	1	0	0	1
r	1	1	1	1	3	5	1	4	9

UDC-system only causes selective dynamic effects, all cells activated from this region regarded as dynamic flexor motor neurones (23 cells in B). Of the other cells 27 were found influenced from NR but not from MesADC and these are regarded as static neurones. The third group in C consists of 13 cells which were tested only from NR and thus could be characterized.

Study of Table II reveals the following interesting points.

The majority of dynamic cells were influenced also from NR. This influence was almost fully reciprocal with excitation of flexor cells and inhibition of extensor cells (B).

Few dynamic cells were not reached by the rubrospinal tract (B).

Among static cells to extensor muscles a mixture of excitation and inhibitory effects was seen while flexor cells were always excited (A).

Also the material of unclassified cells (unknown MesADC-influence) excitatory was on the flexor neurones predominant. Of four extensor cells, on the other hand, all were excited leading to the suggestion that at least these cells were all static, as excitatory cells on extensor dynamic neurones were rare (C).

#### The synaptic linkage between rubrospinal fibres and $\gamma$ -motoneurones

The technique of using long, continuous trains of stimuli in the central area does not allow determination of the latency of the effects evoked. Attempts to use shorter trains of stimuli as often made. From NR it was frequently noted, that cells activated from this region or influenced by trains containing 4 or 5 pulses. This influence, however, had the nature of irregular firing with greatly fluctuating latency from the effective shock and any exact prediction of the segmental latency was impossible.

In one experiment recording was made, not from cell bodies in the lumbar cord, but

TABLE 1 Effects from nucleus ruber (NR) and mesencephalic area for dynamic control of (MesADC) on nine fusimotor neurones in 1 expt. Thresholds for effects given in  $\mu$ A. -, = inhibition, 0 = no influence.

Cell no	Type	Cond. vel.	NR	$\mu$ A	MesADC	$\mu$ A
1	FDL	32 m/s	-	50	+	60
2	DP	32 m/s	+	50	0	70
3	DP	27 m/s	+	60	+	70
4	DP	34 m/s	+	30	+	60
5	DP	27 m/s	+	40	+	70
6	DP	30 m/s	+	40	+	40
7	DP	23 m/s	+	70		
8	PBSI	16 m/s	+	60		
9	FDL		-		0	

Threshold for rubrospinal volley from NR 30  $\mu$ A.

Threshold for rubrospinal volley from MesADC 90  $\mu$ A.

Threshold for PM-response from MesADC 30  $\mu$ A.

effects evoked from the two central regions, while 3 cells were kept only for short time and not fully tested. The threshold for evoking a rubrospinal volley from the just ventrally to the NR was about 30  $\mu$ A. From the electrode placed in MesAI rubrospinal volley could be evoked at stimulating strengths above 90  $\mu$ A. A PM was, on the other hand, evoked already at 30  $\mu$ A. It is clear from the Table that on the different cells were obtained at stimulating intensities which makes spread from one region to the other unlikely. This conclusion is supported by the fact that which was reciprocally influenced from the two regions at low stimulating intensities controlled in the same way but stronger when stimulation was increased to 90  $\mu$ A and to 80  $\mu$ A in MesADC. The central effects on all cells included in the material have been studied under similar circumstances.

A cell influenced in the same reciprocal way as the FDL  $\gamma$  (cell 1) in Table 1 but 1 to the plantaris muscle is illustrated in Fig. 2. This cell was slightly difficult to identify responded antidromically from the nerve together with another cell (A). From the records in A and B it appears, however, that when a spontaneous spike of the cell occurred enough to the antidromic stimulus (B) a collision of orthodromic and antidromic spikes place in the axon and the antidromic response disappeared. The remaining spike belonging to the same nerve was not spontaneously active, nor was it influenced by the stimuli. The rubrospinal volley evoked from the NR-electrode is illustrated in C, characteristic PM-response from the MesADC-electrode in D (note lack of volley in D). In Fig. 2 E is seen a strong inhibitory influence on the spontaneously active cell from the NR at low stimulus intensity. In F the cell is instead shown to be strongly activated from the MesADC. In this experiment the thresholds for detectable descending rubrospinal volley from the NR-electrode and for PM response from the MesADC-electrode were both 40  $\mu$ A. A weak rubrospinal volley for MesADC-stimulation was seen at 100  $\mu$ A.

In Table II the whole material of 63 cells and the effects from MesADC and NR are presented. The cells have been divided into 3 subpopulations on the basis of the effect from MesADC. As previous work on muscle spindle afferents has revealed it

less from NR and MesADC on whole material of 63 cells.

*Influenced from MesADC (Static cells)*

NR	GS	FDL	P	ARSm	PRSt	DP	Extensor	Flexor
	5	0	0	1	9	5	6	14
	5	1	1	0	0	0	7	0
	10	1	1	1	9	5	13	14

*Influenced from MesADC (Dynamic cells)*

NR	GS	FDL	P	ARSm	PRSt	DP	Extensor	Flexor
	1	0	0	5	4		1	9
	3	3	2	0	0		8	0
	4	0	0	0	1		4	1
	8	3	2	5	5		13	10

*Not influenced from MesADC*

NR	FDL	P	ARSm	Tib	PRSt	DP	Ten	Extensor	Flexor
1	1	1	1	1	3	4	1	4	8
1	0	0	0	0	0	1	0	0	1
1	1	1	1	1	3	5	1	4	9

C-system only causes selective dynamic effects, all cells activated from this region are regarded as dynamic fusimotor neurones (23 cells in B). Of the other cells 27 were found influenced from NR but not from MesADC and these are regarded as static neurones. The third group in C consists of 13 cells which were tested only from NR and thus could not be characterized.

Study of Table II reveals the following interesting points.

1. Majority of dynamic cells were influenced also from NR. This influence was almost reciprocal with excitation of flexor cells and inhibition of extensor cells (B).

2. Dynamic cells were not reached by the rubrospinal tract (B).

3. Regarding static cells to extensor muscles—mixture of excitation and inhibitory effects was found while flexor cells were always excited (A).

4. While the material of unclassified cells (unknown MesADC-influence) excitatory on the flexor neurones predominates. Of four extensor cells, on the other hand, all reacted leading to the suggestion that at least these cells were all static, as excitatory on extensor dynamic neurones were rare (C).

#### *Asynchronous linkage between rubrospinal fibres and $\gamma$ -motoneurones*

The technique of using long, continuous trains of stimuli in the central areas does not allow determination of the latency of the effects evoked. Attempts to use shorter trains of stimuli were often made. From NR it was frequently noted, that cells activated from this region were influenced by trains containing 4 or 5 pulses. This influence, however, had the nature of irregular firing with greatly fluctuating latency from the effective shock and any exact determination of the segmental latency was impossible.

In one experiment recording was made, not from cell bodies in the lumbar cord, but

TABLE I Effects from nucleus ruber (NR) and mesencephalic area for dynamic control of (MesADC) on nine fusimotor neurones in 1 expt. Thresholds for effects given in  $\mu$ A. — = inhibition, 0 = no influence.

Cell no.	Type	Cond. vel.	NR	$\mu$ A	MesADC	$\mu$ A
1	FDL	32 m/s	—	50	+	60
2	DP	32 m/s	+	50	0	70
3	DP	27 m/s	+	60	+	70
4	DP	34 m/s	+	30	+	60
5	DP	27 m/s	+	40	+	70
6	DP	30 m/s	+	40	+	40
7	DP	23 m/s	+	70		
8	PBSI	16 m/s	+	60		
9	FDL		—		0	

Threshold for rubrospinal volley from NR 30  $\mu$ A.

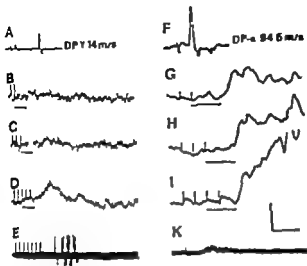
Threshold for rubrospinal volley from MesADC 90  $\mu$ A.

Threshold for PM response from MesADC 30  $\mu$ A.

effects evoked from the two central regions, while 3 cells were kept only for short time and not fully tested. The threshold for evoking a rubrospinal volley from D just ventrally to the NR was about 30  $\mu$ A. From the electrode placed in MesA rubrospinal volley could be evoked at stimulating strengths above 90  $\mu$ A. A P was, on the other hand, evoked already at 30  $\mu$ A. It is clear from the Table that on the different cells were obtained at stimulating intensities which makes spread from one region to the other unlikely. This conclusion is supported by the fact that which was reciprocally influenced from the two regions at low stimulating intensity controlled in the same way but stronger when stimulation was increased to 90 and to 80  $\mu$ A in MesADC. The central effects on all cells included in the material have been studied under similar circumstances.

A cell influenced in the same reciprocal way as the FDL  $\gamma$  (cell 1) in Table I but to the plantaris muscle is illustrated in Fig. 2. This cell was slightly difficult to identify and responded antidromically from the nerve together with another cell (A). From the 1 in A and B it appears, however, that when a spontaneous spike of the cell occurred enough to the antidromic stimulus (B) a collision of orthodromic and antidromic spikes place in the axon and the antidromic response disappeared. The remaining spikes belonging to the same nerve was not spontaneously active, nor was it influenced by the stimuli. The rubrospinal volley evoked from the NR-electrode is illustrated in C, characteristic PM-response from the MesADC-electrode in D (note lack of volley in B). In Fig. 2 E is seen a strong inhibitory influence on the spontaneously active cell F at low stimulus intensity. In F the cell is instead shown to be strongly activated from ADC. In this experiment the thresholds for detectable descending rubrospinal volley from the NR-electrode and for PM-response from the MesADC-electrode were both 40  $\mu$ A. A weak rubrospinal volley for MesADC-stimulation was seen at 100  $\mu$ A.

In Table II the whole material of 11 cells and the effects from MesADC and NR are presented. The cells have been divided into 3 subpopulations on the basis of the effects from MesADC. As previous work on muscle spindle afferents has revealed 1



Intracellular recording from flexor motor (A-E) and intracellular recording from skeleto-motor neuron both belonging to the deep peroneal nerve. In A and F the cells are antidromically identified. B and G-I postsynaptic potentials caused by varying number of stimuli in NR are shown. The horizontal line below each record denotes the distance between the electro-stimulus and the recording. In E the flexor motor neuron is seen to start firing (superimposed records) to an increased number of stimuli. In K is shown the descending rubrospinal volley in  $L_4$ . Voltage calibration is 4 mV for A, 9.4 mV for B-D, 10 mV for F-I. Time: 10 ms for B-E, 4 ms for A and F-K.

In this experiment reached the L7 segment in 3.1 ms (Fig. 4 K shows the volley led in L5 with a latency of 2.9 ms. About 0.2 ms should be added to account for the focal distance of 15 mm to the L7 segment). This leads to about 1.2 and 1.9 ms synaptic delay for the  $\alpha$ - and the  $\gamma$ -cell respectively. Also this observation indicates that a disynaptic link between the rubrospinal tract and  $\alpha$ - as well as static  $\gamma$ -cells may occur (cf. Discussion). During the course of the experiments a considerable number of intracellular recordings of  $\alpha$ -motoneurons were obtained. On each such occasion the effect on the cell from the stimulating electrode as well as from the MesADC was tested. It was consistently found that while the rubrospinal activation regularly caused postsynaptic potentials in these  $\alpha$ -motoneurons according to the pattern described by Hongo *et al.* (1969 a)  $\alpha$ -motoneurons were never excited in any respect from the MesADC (cf. Discussion).

#### Conduction velocities of static and dynamic axons

In the whole material of 53 cells the conduction velocities of their axons varied from 0.35 m/s. For the 23 cells classified as dynamic the axon conduction velocity range was 0.7 m/s with a mean of 31.1 m/s, and for 26 static cells the corresponding figures were 35 m/s and 27.5 m/s. The tendency for the lowest conduction velocities to be found in static neurons and the highest in dynamic ones is clear but not statistically significant. It is, however, partly in agreement with the observations made by Brown, Crowe and Matthews (1965) who for the tibialis anterior muscle found that the slowest  $\gamma$ -fibres (in their study with conduction velocities below 30 m/s) were virtually certain to be static. For the soleus muscle, on the other hand, Crowe and Matthews (1964) found no such clear differences in conduction

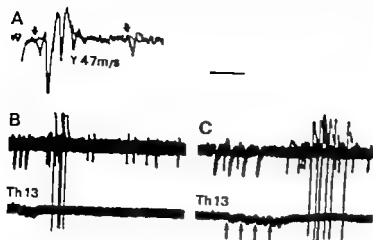


Fig. 3. A, Identification of stimulus formotor neurone in nerve filament. In B three and four shocks activate  $\alpha$  as well as  $\gamma$  neurones (upper traces). In C inhibitory volley recorded in Th<sub>13</sub> (lower traces). For further see text. Time calibration for A, 10 ms for B and 4 ms

instead from axons in branches of the tenuissimus nerve. In this experiment a nerve fil was obtained containing a  $\gamma$ -fibre with a conduction velocity of 47 m/s (Fig. 3 A). The was spontaneously active (cf arrows in A). The filament also contained some  $\alpha$ -axons of these were activated together with the  $\gamma$ -fibre from the L7 ventral root (Fig. 3 A), a belonged to another ventral root. The  $\gamma$ -fibre as well as the unidentified  $\alpha$ -fibre were activated from NR by a short train of stimuli. From the analysis of a number of imposed records of the type illustrated in Fig. 3 B and C it became clear that the number of pulses required in the NR to activate the  $\gamma$ -axon varied from record to record. Our measurements of intervals between different stimulating pulses and the resulting burst spikes did, however reveal that the minimal latency from active pulse to fired  $\gamma$ -spike was 7.8 ms. (In seven records each consisting of 10 or 20 superimposed sweeps this latency was found from the second pulse in four cases and from the first, the third or fourth pulse in one case each). In Fig. 3 B and C the cell is in both cases fired by the second rubral pulse. In this experiment activity in the fastest rubrospinal fibres reached the T1 segment in 2.0 ms (cf arrows in Fig. 3 C, lower trace) and the L7-segment in 2.5 ms (additional distance 60 mm). Thus 5.3 ms remains for segmental synaptic delay and central and peripheral conduction. Conduction through the ventral root and the nerve could be calculated on the basis of the known conduction velocity of the fibre and the measured distance and was 3.5 ms. This leaves a segmental delay of 1.8 ms for the activation of this  $\gamma$ -unit. It seems to imply a disynaptic linkage from the rubrospinal tract to this unit (cf Discussion).

More conclusive evidence concerning the synaptic linkage between the rubrospinal tract and  $\alpha$  as well as  $\gamma$ -motoneurons was obtained in recordings from cells belonging to the DP-nerve in another experiment (Fig. 4). In both cells, the  $\alpha$  conducting at 94.5 m/s and the  $\gamma$  (classified as static and probably being recorded from in a juxtacellular position) at 14 m/s occasional EPSPs could be evoked by two NR-pulses (B and G). Usually however three pulses were required to regularly depolarize the cells (C and H). When four or five stimuli were used these caused additional depolarization by their EPSPs summing to the one caused by the third pulse (D and J). At still longer trains the  $\gamma$ -cell started to fire as illustrated by the superimposed record in E. The latency from the active stimulating pulse to the resulting EPSP was for the  $\alpha$ -cell 4.3 ms, for the  $\gamma$ -cell 5.0 ms. The descending rubrospinal



in two functional classes, static and dynamic cells. The method adopted for this was upon previous findings that the MesADC-region, *i.e.* the area just dorsally to and lying into the dorsal part of the red nucleus, selectively influences the dynamic sensitivity of muscle spindles (*cf.* Appelberg 1967, Appelberg and Jäneskog 1972 and also Appelberg and Enander-Densén 1965 who showed that effects on extensor as well as flexor spindles derived from the same area). The work of Appelberg and Jäneskog (1972) is of special interest in this connection. During their experiments various types of influences on single motoneurons could be observed from different levels in a stimulating electrode track passing through the mesencephalon at a rubral level. It became quite clear that the effects evoked from the area just dorsally to the red nucleus were always purely dynamic (in contrast to those evoked from sites within the red nucleus, which were often mixed static/dynamic and, in light of the present experiments, probably at times evoked via the rubrospinal tract). Jäneskog (1974a) later made a detailed comparison between the mesencephalic area evoking dynamic spindic effects and the one evoking a D-zone response in the cerebellar vermal lobule and found a near to perfect coincidence between the two. In the present experiments the MesADC-electrode was placed with such a PM-response as a guide. At the same time it was carefully controlled that no rubrospinal descending volley was evoked from the chosen electrode site. With all this in mind we feel it fully justified to use such a MesADC-stimulus as a methodological tool enabling the separation of  $\gamma$ -motoneurons into two functional classes: static and dynamic.

Applying the method of classification leads to an important conclusion, namely that dynamic  $\gamma$ -motoneurons are also controlled by the rubrospinal tract. From this we infer that the rubrospinal system may be thought of as a motor system working with both static and possibly both classes of  $\gamma$ -motoneurons. Nothing is known, however, about the synaptic coupling between the tract and dynamic neurones. It may be of some relevance also to note that of 23 cells classified as dynamic 5 were not influenced by the NR (Table II B). This may indicate that there exists a population of dynamic  $\gamma$ -motoneurons not participating in  $\alpha$ - $\gamma$ -linked control but rather being private to the specialized dynamic control system. Whether other such subpopulations of dynamic  $\gamma$ -motoneurons exist possibly being private to other descending or reflex systems is at present not known. Appelberg and Jäneskog (1972) did, however, note that not all spindles seemed to have their dynamic sensitivity controlled by the MesADC system. It should finally be pointed out, that the observations of lack of any effect on  $\alpha$ -motoneurons from the MesADC (while synaptic potentials were readily evoked from the NR) are of considerable importance. They do strengthen the notion that the MesADC-system, according to Jäneskog (1974a, b) may be a rubro-bulbo-spinal system with its origin in the rostral parts of the NR, is a system exclusively controlling one type of motoneurons, the dynamic fusimotor ones. Whether this system may also have other neuronal connections to the spinal cord is not known. In fact a descending volley reminding of the one caused by the dorsal reticulo-spinal system (Baldessarini *et al.* 1972a) was sometimes observed when stimulating the MesADC. The possible identity of the MesADC-system and the dorsal reticulo-spinal system remains an object for further investigation.

This work was supported by the Swedish Medical Research Council, Project No. 3873.

velocities between static and dynamic  $\gamma$ -axons. With such differences between individual muscles it is clear that the matter of conduction velocities should not be too much sized in a material obtained from several muscles.

### Discussion

The primary aim of the present investigation was to study the effects of the rubro tract on  $\gamma$ -motoneurons. According to Baldissera *et al* (1972 a and b) segmental rubro effects may be studied in isolation after a bilateral ablation of the sensory-motor cort. the stimulating electrode is placed so as to activate interposito-rubral fibres just ventral to the NR. These precautions were taken in the present experiments and it seems safe to conclude that the effects studied were truly rubrospinal. Within the whole material of 30 extensor cells 11 were excited, 15 inhibited and 4 not influenced from the NR. Of 33 flexor cells 10 were excited, 1 inhibited and 1 not influenced. In a corresponding material of intracellularly recorded  $\alpha$ -motoneurons (Hongo *et al* 1969 a, their Table 1) 45 out of 57 flexor  $\alpha$ -motoneurons were purely excited, none were purely inhibited while 12 received a mixed excitation and inhibition. Of 125 extensor motoneurons 50 were excited, 37 inhibited and 38 influenced in a mixed way. Although our material is smaller and obtained by extracellular recording it is immediately evident that there is a close correspondence in the pattern of rubrospinal control of  $\alpha$ - and  $\gamma$ -motoneurons.

Having in mind the concept of  $\alpha$ - $\gamma$ -linkage it is, however, clearly not enough to demonstrate a similarity in effect on the two groups of motoneurons from a certain source. It is also necessary to demonstrate an equality in minimal synaptic linkage. That the rubrospinal tract fibres may contact  $\alpha$ -motoneurons via a single interneurone seems well established (Hongo *et al* 1969 a and verified in the present investigation).

In the present work on  $\gamma$ -motoneurons, on the other hand, careful determination of the central delay was arrived at only twice. For the juxtacellularly recorded DP-cell this delay was 1.9 ms, a figure possibly allowing a passage through three synapses. It is possible, however, to suggest a disynaptic linkage and ascribe the seemingly long delay to the possibility that the descending fibre activating the cell had a somewhat slower conduction velocity than the fastest ones causing the very beginning of the descending volley.

For the  $\gamma$ -fibre recorded in the tenuissimus muscle nerve the central delay could be calculated with satisfactory accuracy to be 1.8 ms. In this case, however, the time arrived includes spike initiation and the disynaptic nature of the coupling can hardly be doubted. The fact that the cell in one recording actually responded to the first shock in NR may be attributed to the fact that the cell was spontaneously active and therefore sometimes extremely easily triggered.

In conclusion it is, therefore, provisionally suggested that the synaptic coupling between the rubrospinal tract and  $\alpha$ - as well as static  $\gamma$ -motoneurons is minimally disynaptic. The rubrospinal tract is added to the list of descending systems theoretically capable of providing a  $\gamma$ -linked movement. The vestibulo-spinal tract as well as the MLF-system were previously shown to be organized in this manner (*cf* Grillner 1969).

The second aim of the experiments was to attempt to divide the material of  $\gamma$ -motoneurons

## A Method for the Continuous Study of Net Water Transport in the Feline Small Bowel

By

MATS JODAL, DAN-AXEL HALLBÄCK, JOAR SVANVİK and OVE LUNDGREN

Received 22 May 1975

### Abstract

M. JODAL, D.-A. HALLBÄCK, J. SVANVİK and O. LUNDGREN. *A method for the continuous study of net water transport in the feline small bowel* Acta physiol. scand. 1975 95 441-7

A new technique has been developed for the study of net water transport across the intestinal mucosa. The lumen of an isolated intestinal segment is steadily perfused with solution of known osmolarity in closed perfusion system with reservoir large enough to prevent recirculation. The osmolarity may be monitored on plethysmograph. Changes in the perfused volume is recorded by a transducer coupled to the recirculating system via T-tube. If no motility occurs, the changes of the volume reflects net water transport across the intestinal epithelium. A quantitative comparison of the technique with the conventional polyethylene glycol method revealed no significant difference. The new technique also makes it possible to quantify the net water absorption via lymph and blood.

A number of methods have been developed for the study of net water transport across intestinal epithelium in animals and man (for review see Parsons 1968). One of the most common techniques involves the use of a solution containing an inert, nonabsorbable tracer, often polyethylene glycol. The solution is infused in one part of the intestine and collected downstream. An increase (decrease) of the concentration of the inert tracer during passage through the intestine reflects a net water absorption (secretion), which can be calculated. Adding radioactively labelled water to the perfusion medium also enables the study of unidirectional fluxes of water across the gut epithelium. However with this method it is difficult to follow transient changes of net water absorption occurring within minutes, it is necessary to perfuse the intestinal segment during a fairly long time period to reach a steady state situation, as regards the tracer. To circumvent these difficulties in the anesthetized animal, a perfusion method has been developed which allows a continuous study of net water transport across the epithelium of the feline small bowel.

### Methods

*Preparation procedures and determination of blood flow.* Experiments were performed on 23 cats of both sexes weighing 2-4 kg, anaesthetized intravenously with pentobarbital (5 mg/kg b.w.) after induction with chloral hydrate (0.5 g/kg b.w.).

## References

- APPELBERG, B., The effect of electrical stimulation in nucleus ruber on the response to stretch in  $\alpha$  and secondary muscle spindle afferents. *Acta physiol. scand.* 1962, 56, 140-151.
- APPELBERG, B., A rubro-olivary pathway II Simultaneous action on dynamic fusimotor neurones and activity of the posterior lobe of the cerebellar cortex. *Exp. Brain Res.* 1967 3, 382-390.
- APPELBERG, B. and F. EMOND-DENAND, Central control of static and dynamic sensitivities of spindle primary endings. *Acta physiol. scand.* 1965 63, 487-494.
- APPELBERG, B. and I. Z. KOSARY, Excitation of flexor fusimotor neurones by electrical stimulation of nucleus ruber. *Acta physiol. scand.* 1963 59, 445-453.
- APPELBERG, B. and T. JENESKÖI, Mesencephalic fusimotor control. *Exp. Brain Res.* 1971, 15, 97-110.
- BALDWIN, F. A. LUNDBERG and M. UDO, Activity evoked from the mesencephalic tegmentum ascending pathways other than the rubrospinal tract. *Exp. Brain Res.* 1972 a, 15, 133-150.
- BALDWIN, F. A. LUNDBERG and M. UDO, Stimulation of pre- and postsynaptic elements in the nucleus. *Exp. Brain Res.* 1972 b, 15, 151-167.
- BROWN, M. C. A. CROWE and P. B. C. MATTHEWS, Observations on the fusimotor fibres of the posterior muscle of the cat. *J. Physiol. (Lond.)* 1965, 177, 140-159.
- CROWE, A. and P. B. C. MATTHEWS, Further studies of static and dynamic fusimotor fibres. *J. Physiol. (Lond.)* 1964, 174, 132-151.
- GRILLNER, S., Spinal and segmental control of static and dynamic  $\gamma$ -motoneurons in the cat. *Physiol. scand.* 1969 Suppl. 327.
- HOMO, T., ELŻBIETA JANKOWSKA and A. LUNDBERG, The rubrospinal tract. I. Effects on alpha motoneurons innervating hindlimb muscles of the cat. *Exp. Brain Res.* 1969 a, 7, 344-364.
- HOMO, T., ELŻBIETA JANKOWSKA and A. LUNDBERG, The rubrospinal tract. II. Facilitation of interneuronal transmission in reflex paths to motoneurons. *Exp. Brain Res.* 1969 b, 7, 365-391.
- HOMO, T., ELŻBIETA JANKOWSKA and A. LUNDBERG, The rubrospinal tract. III. Effects on presynaptic terminals. *Exp. Brain Res.* 1972 a, 15, 39-53.
- HOMO, T., ELŻBIETA JANKOWSKA and A. LUNDBERG, The rubrospinal tract. IV. Effects on interneurons. *Exp. Brain Res.* 1972 b, 15, 54-78.
- JENESKÖI, T., Parallel activation of dynamic fusimotor neurones and a climbing fibre system from brain stem. I. Effects from the rubral region. *Acta physiol. scand.* 1974 a, 91, 223-242.
- JENESKÖI, T., Parallel activation of dynamic fusimotor neurones and a climbing fibre system from brain stem. II. Effects from the inferior olivary region. *Acta physiol. scand.* 1974 b, 92, 66-83.
- SASAKI, K., A. NAMIKAWA and S. HASHIRAMOTO, The effect of midbrain stimulation upon alpha motoneurons in lumbar spinal cord of the cat. *Jap. J. Physiol.* 1960, 10, 303-316.

ed 2), according to Grass polygraph. Vessel outflow pressure could be set at any desired level of the end of the tube draining the drop recorder. It was usually kept around 10 mm as blood was returned to the animal via a funnel and catheter in the right jugular vein. In the most lymph vessel draining the intestinal segment and its lymph nodes as cannulated easily. In the rest of the experiments the lymph vessels were left untouched or cut open. It was carefully avoided during the operative procedures and, if occurring, substitution by L. body-saline Dextran-Tyrode solution was performed. Body temperature was maintained  $37^{\circ}\text{C}$  by means of thermostatically regulated heating pad.

and plethysmographic techniques. In all experiments the jejunal lumen was perfused at a solution of known composition (see below). To make this possible the jejunal segment (in recirculating system with a large peristaltic reservoir containing about 900 ml of fluid) the fluid was perfused at a constant rate (usually around 1 ml/min) through the intestinal lumen of a solid pump (Hematec 5a, Zürich, Switzerland, Model mp-ge), the temperature of the fluid entering the segment being continuously recorded by thermocouple thermometer (Copenhagen) and kept at  $37^{\circ}\text{C}$  by heating pad. To prevent mixing in the reservoir and, moreover, the peristaltic reservoir was divided into number of interconnected smaller compartments. The recirculation system was connected to volume transducer as T-tube, continuously emptying at the recirculation volume (Fig. 1 and 2). The pressure at the outflow tubing could be measured at the height of the volume transducer procedure such influenced net water absorption (Dowling and Habrecht 1971). The outflow pressure was usually kept at the lower end of physiological,  $1-1.2$  cm  $\text{H}_2\text{O}$ .

specimens to the intestinal segment was also enclosed in a triangular peristaltic plethysmograph technique and measure recording of the volume of the jejunal segment and its lymph nodes it was made possible by placing the widebore cannula in the mesenteric vein at the proximal end of the plethysmograph, thereby avoiding venous collapse by undue lateral pressure (Follett). The opening in the plethysmograph was sealed by silicone grease (Dow Corning). The plethysmograph was filled with modified Krebs-Henseleit solution (see below) and connected to a plethysmograph on Grass polygraph. The temperature of the plethysmograph was maintained at  $37^{\circ}\text{C}$ , only controlled by thermocouple thermometer (Electrolab, Copenhagen). The plethysmograph, isolating the jejunal segment to the perfusion system fitted closely through openings in the plethysmograph wall, making it possible to perfuse the lumen of the intestinal segment in *in vivo* plethysmograph.

Net water transport was also performed in which the intestinal segment was not enclosed in the plethysmograph. The set up was then otherwise identical to that described above for the plethysmographic (Fig. 2).

polyethylene glycol method. Net water transport was in some experiments determined by means of polyethylene glycol (PEG) molecular weight around 4000; purchased from New England Nuclear, Boston, Mass. or from Radiochemical Centre, Amersham, England). To 100 ml of the solution 0.5  $\mu\text{Ci}$  of PEG was added. When the perfusion through the intestinal segment had been running "steady state" for such long time that the perfused volume amounted to at least 10 volumes assumed to be contained in the gut lumen, 200  $\mu\text{l}$  samples were taken from the inflow and outflow of the intestinal segment. After adding 8 ml of Instagel<sup>®</sup> (Packard), radioactivity was measured.

Packard Tricarb Liquid Scintillation Spectrometer, model 3120 (counting efficiency around 80%, quenching being controlled by the external standard and/or the channel ratio method). The rate of the recirculation system was checked before and after each experimental run. Net water transport was calculated from the change of concentration of the tracer during its passage through the

and bicarbonate balance of the experimental animal. In order to maintain fairly constant arterial pH throughout the experiments, bicarbonate solution (10 mmol  $\text{NaHCO}_3$  per 100 ml 0.9% glucose) was infused at a rate of 0.1 ml/min during the experiments. This procedure was earlier described to maintain arterial pH and  $\text{Pco}_2$  at normal and constant level, despite the surgical trauma (Lundgren 1972).

showed. In most experiments the perfusion solution contained (mmoles/l, pro analysis):  $\text{NaCl}$  122,  $\text{NaHCO}_3$  25,  $\text{KH}_2\text{P}_2\text{O}_7$  1.2,  $\text{MgCl}_2$  64,  $\text{CaCl}_2$  1.2, Glucose 30,  $\text{CaCl}_2$  2.5. Osmolality of the solution was 316 mOsm/kg  $\text{H}_2\text{O}$  as determined by an Advanced Instruments osmometer and plasma osmolality was of the same order of magnitude ( $316 \pm 10$  mOsm/kg). If plasma osmolality exceeded 330, osmolality of the perfusion solution was adjusted to plasma osmolality by the addition of appropriate

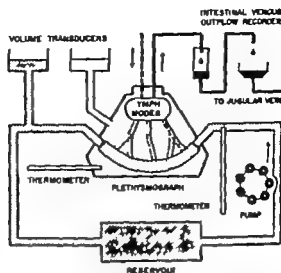


Fig. 1 Schematic illustration of the plethysmographic technique used for studying net water across the intestinal epithelium as well as net water absorption in lymph and blood.

either. The cats had been deprived of food for 24 h with free access to water and exhibited no obvious signs of intestinal disease.

The operative procedures were largely similar to those used in previous studies on the intestine in this laboratory (cf Folkow *et al.* 1963, Karpp *et al.* 1968). The abdomen was opened in the midline and the greater omentum and the spleen were excised. A segment of the jejunum, about 10 cm, was isolated and the remainder of the intestinal tract was ligated. The lumen of the jejunal segment was flushed with bodywarm saline until the leaving fluid was clear. Care was taken to keep the intestinal segment moistened throughout the operative procedures by gauze soaked in saline. The influence of the sympathico-adrenal system was eliminated by bilateral ligation of the splanchnic nerves, denervating also the adrenal glands. The vagal influence was eliminated by administering atropine, 1 mg/kg b.w.

After heparinization the right femoral artery was connected to a Statam pressure transducer to record mean arterial pressure. The superior mesenteric vein draining all blood from the jejunum and its lymph nodes, was cannulated and connected to an optical drop recorder unit operating at

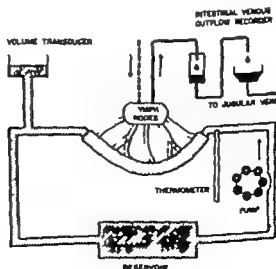


Fig. 2 Schematic illustration of the perfusion system used for recording net water transport in the intestine.

(Fig. 1 and 2), recording on Grass polygraph. Venous outflow pressure could be set at any desired by adjustment of the end of the tube draining the drop recorder. It was usually kept around 10 mm. The venous blood was returned to the animal as femoral and catheter in the right jugular vein. In experiments the main lymph vessel draining the intestinal segment and its lymph nodes was cannulated and as possible. In the rest of the experiments the lymph vessels were left untouched or cut open. Ligation was carefully avoided during the operative procedures and, if occurring, substitution by 1 mm of body-warm Dextran-Tyrosine solution was performed. Body temperature was maintained at 37°C by means of thermostatically regulated heating pad.

*Perfusion and plethysmographic techniques.* In all experiments the jejunal lumen as perfused at constant rate with solution of known composition (see below). To make this possible the jejunal segment enclosed in recirculating system with large peristaltic reservoir containing about 900 ml of fluid (Fig. 2). The fluid is perfused at constant rate (usually around 1 ml/min) through the intestinal lumen by means of roller pump (Bioscience, Zürich, Switzerland, Model 80-30), the temperature of perfused fluid entering the segment being continuously recorded by thermocouple thermometer (Czech, Copenhagen) and kept at 38°C by heating pad. To promote mixing in the reservoir and, as needed, the peristaltic reservoir was divided into number of interconnected smaller chambers. The recirculation system was connected to volume transducer via T-tube, continuously being changed in the recirculation volume (Fig. 1 and 2). The pressure at the outflow tubing could be set by adjustment of the height of the volume transducer, procedure which influenced net water absorption (Donaldson and Habrecht 1973). The outflow pressure was usually kept at the lower end of physiological range, i.e. 1-2 cm H<sub>2</sub>O.

Intestinally the intestinal segment was also enclosed in triangular peristaltic plethysmograph for continuous and sensitive recording of the volume of the jejunal segment and its lymph nodes (Fig. 2). This was made possible by placing the plethysmograph in the mesenteric vein at the proximal end of the plethysmograph, thereby avoiding venous collapse by undue lateral pressure (Folkow 1969). The opening in the plethysmograph was sealed by adhesive grease (Dow Corning). The plethysmograph was filled with modified Krebs-Henseleit solution (see below) and connected to a volume transducer as Grass polygraph. The temperature of the plethysmograph was maintained at 38°C, continuously measured by thermocouple thermometer (Electrolab, Copenhagen). The polyethylene tubing connecting the jejunal segment to the perfusion system fitted closely through openings in the plethysmograph wall, making it possible to perfuse the lumen of the intestinal segment in situ inside plethysmograph.

Experiments were also performed in which the intestinal segment was not enclosed in the plethysmograph. In this case the set up was then otherwise identical to that described above for the plethysmographic technique (Fig. 2).

*<sup>14</sup>C-Polyethylene glycol method.* Net water transport was in some experiments determined by means of <sup>14</sup>C-polyethylene glycol (PEG) molecular weight around 4000; purchased from New England Nuclear Inc., Boston, Mass. or from Radiochemical Centre, Amersham, England. To 100 ml of the perfusion solution 0.5 µCi of PEG was added. When the perfusion through the intestinal segment had been allowed during "steady state" for each long time that the perfused volume amounted to at least 10 ml the volume assumed to be contained in the gut lumen, 200 µl samples were taken from the outflow at the outflow of the intestinal segment. After adding 8 ml of Instagel® (Packard), radioactivity was measured in Packard Tri Carb Liquid Scintillation Spectrometer model 3320 (counting efficiency around 15% for <sup>14</sup>C), quenching being controlled by the external standard and/or the channel ratio method. The count rate of the recirculation system was checked before and after each experimental run. Net water transport was calculated from the change of concentration of the tracer during its passage through the intestine.

*pH and base balance of the experimental animal.* In order to maintain fairly constant arterial pH and P<sub>CO</sub><sub>2</sub> throughout the experiments, bicarbonate solution (10 mmol NaHCO<sub>3</sub> per 100 ml 10% glucose solution) was infused at rate of 0.1 ml/min during the experiments. This procedure was earlier demonstrated to maintain arterial pH and P<sub>CO</sub><sub>2</sub> at normal and constant level, despite the surgical trauma (Björk and Lundgren 1972).

*Solutions.* In most experiments the perfusion solution contained (mmoles/l, pro volu): NaCl 122, CaCl<sub>2</sub> 2.5, MgCl<sub>2</sub> 1.2, KCl 1.2, H<sub>2</sub>O 1.2, Glucose 30, CaCl<sub>2</sub> 2.5. Osmolality of this solution was around 310 mOsm. 1g H<sub>2</sub>O is determined by an Advanced Instruments' osmometer and plasma osmolality was usually of the same order of magnitude (316 ± 10 mOsm/kg). If plasma osmolality exceeded 320, the osmolality of the perfusion solution was adjusted to plasma osmolality by the addition of appropriate

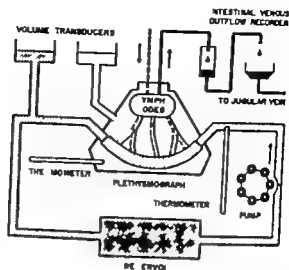


Fig. 1. Schematic illustration of the plethysmographic technique used for studying net water transport across the intestinal epithelium as well as net water absorption in lymph and blood.

either. The cats had been deprived of food for 24 h with free access to water and exhibited no signs of intestinal disease.

The operative procedures were largely similar to those used in previous studies on the intestine in this laboratory (cf. Folkow *et al.* 1963; Karpp *et al.* 1968). The abdomen was opened up and the greater omentum and the spleen were extirpated. A segment of the jejunum, about 10 cm, the Treitz's ligament and weighing 10–20 g, was isolated and the remainder of the intestinal tract was flushed with bodywarm saline until the leaving fluid was clear. The lumen of the jejunal segment was flushed with bodywarm saline until the leaving fluid was clear. The influence of the sympathetic-adrenal system was eliminated by blockade of the splanchnic nerves, denervating also the adrenal glands. The vagal influence was eliminated by administering atropine, 1 mg/kg b.w.

After heparinization the right femoral artery was connected to a Statam pressure transducer to record mean arterial pressure. The superior mesenteric vein draining all blood from the jejunum and its lymph nodes, was cannulated and connected to an optical drop recorder and operating at

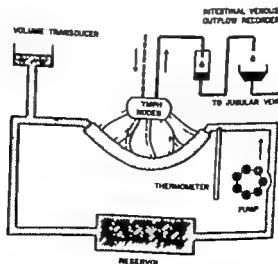
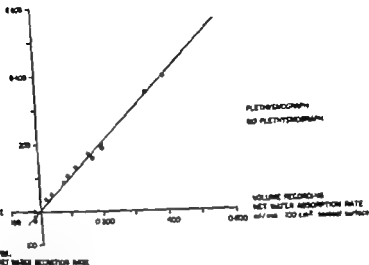


Fig. 2. Schematic illustration of the perfusion system used for recording net water transport in the intestine of the cat.



PEL.  
NET WATER ABSORPTION RATE  
ml/min 100 cm<sup>2</sup> exposed surface



PEL.  
NET WATER ABSORPTION RATE

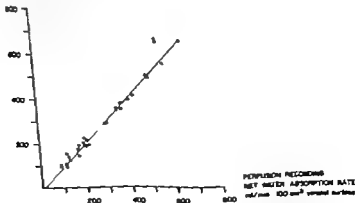
Fig. 4 Correlation between net water transport rate determined by means of polyethylene glycol (PEG) and by the previous methods depicted in Fig. 1 and 2. Line denotes eq.  $y = 0.953x - 0.007$  determined by a method of least squares.  $r = 0.94$  ( $n = 33$ ).

CHANGE OF PLETHYSMOGRAPHIC VOLUME



Fig. 5 The mode of analysis of the plethysmographic recording, schematically illustrated. At arrow the plethysmographic volume of the perfusion system (see Fig. 1) was clamped. For details, see text!

PLETHYSMOGRAPHIC RECORDING  
NET WATER ABSORPTION RATE  
ml/min 100 cm<sup>2</sup> exposed surface



PERFUSION RECORDING  
NET WATER ABSORPTION RATE  
ml/min 100 cm<sup>2</sup> exposed surface

Fig. 6 The correlation between net water absorption rate determined from the decline of perfusion volume and calculated from the change in plethysmographic volume (see text). Line denotes eq.  $y = 1.069x - 0.01$  calculated by the method of least squares.  $r = 0.98$  ( $n = 41$ ).

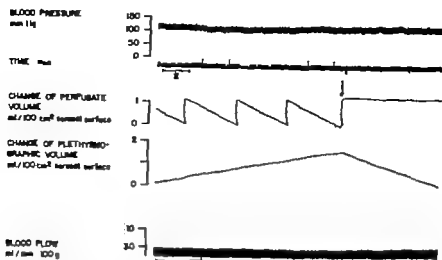


Fig. 3 Original tracing from experiment using the plethysmographic method of Fig. 1. "Start" denotes addition of 0.5 ml to the volume transducer of the perfusion system (left transducer of Fig. 1). At a volume transducer of the perfusion system was excluded from the recirculating system by means of Péan's forceps.

amounts of mannitol. The solution in the plethysmograph was identical to the above-mentioned one in that no  $\text{CaCl}_2$  was added. In some experiments 5–10  $\mu\text{g}$  of isoprenaline was added to the plethysmograph solution (200 ml) to minimize motility.

## Results

The rate of net water absorption (or secretion) was in all experiments estimated from continuous decrease of perfusate volume, recorded by the volume transducer as shown in the second upper panel of Fig. 3. In 13 expts. the rate of net water transport recorded by this technique was compared with that estimated using  $^{14}\text{C}$  PEG. The results of these experiments demonstrated a close agreement between the two methods, as illustrated in Fig. 4.

In the plethysmographic experiments, an increase of the volume contained within the plethysmograph was regularly noted when the intestine was in the absorptive state. When absorption occurred, no increase of plethysmographic volume was recorded. However, excluding the left volume transducer of Fig. 1 from the recirculating system by a Péan's forceps reversed the increase of plethysmographic volume to a decrease as depicted on Fig. 3 (arrow denotes clamping).

It was felt that the increase of plethysmographic volume illustrated in the left part of Fig. 3 was related to the absorption of water via the lymphatic vessels and that the decrease in the right part of Fig. 3 reflected net blood absorption. For this reason, the plethysmographic recordings were analyzed as shown in Fig. 5. The rate of volume increase ( $\Delta V_i$ ) recorded prior to clamping was added to the rate of volume decrease ( $\Delta V_d$ ) seen after clamping. The sum of these two volume changes was compared to the simultaneously recorded net water absorption rate, calculated from the change of perfusate volume. Such a comparison, formed in 18 experiments at different levels of absorption rate, revealed a close correlation between the two methods as illustrated in Fig. 6.

Furthermore, in some plethysmographic experiments it was possible to cannulate on



TABLE 1 Comparison between lymph flow expressed in ml/min, estimated from change of plethysmographic volume and from volume of lymph collected *via* a lymphatic cannula.

Plethysmographic recording	Collected lymph
0.0248	0.0239
0.0096	0.0113
0.0016	0.0015
0.0017	0.0014

the lymphatic vessels draining the intestine and its lymph nodes. Lymph flow could then be determined by collecting lymph during a 15–20 min period. This flow value was compared with that determined from the increase of plethysmographic volumes seen after occluding the lymph cannula. The data obtained in 4 experiments are shown in Table 1. No significant difference between the two sets of values was found.

Quantitatively net water absorption rate at "resting" intestinal blood flow amounted to 0.25–0.50 ml/min/100 cm serosal surface, when the intestine was isovolumetric and net luminal pressure at the outflow end of the intestine was 1–2 cm H<sub>2</sub>O. 20–40% of this volume was absorbed *via* the lymphatic vessels. A detailed quantitative account of these experiments will be given in a subsequent report.

### Discussion

This paper presents a simple method making it possible to study continuously the net transport of water across the intestinal epithelium of an anesthetized animal. A comparison between this method and the conventional PEG-technique revealed a close correlation (Fig. 4). The major technical difficulty of the present technique is the presence of motility, which was easily detected by inspection and/or by abrupt volume changes of the recirculating perfusate volume. To avoid motility various precautions were taken. First, the segment used in the experiments was a jejunal one situated about 10 cm below Treitz's ligament to avoid the pacemaker regions in the most proximal part of the gut. Second, intraluminal pressure was kept at the lower end of the physiological range, i.e. at a pressure of 1–2 cm H<sub>2</sub>O at the outflow end. Third, the plethysmographic solution was devoid of Ca, the presence of which is necessary for the initiation of smooth muscle contractions. Fourth, in so-called plethysmographic experiments, a small amount of isoprenaline, a potent smooth muscle relaxing agent, was added. Fifth, great care was taken to keep the segment moistened and prevent cooling of the segment. With these precautions taken, the intestine exhibited motility in most experiments.

It was regularly noted that the plethysmographic volume recording increased when water absorption occurred. In situations when one may assume that the intestinal tissue is "isovolumetric" this increase in all probability reflects the absorption of water *via* lymphatic vessels. This fluid absorbed was then "trapped" in the plethysmograph since it could not overcome the high sealing pressure at the plethysmographic exit. Since the lymphatic vessels of the cat mesentery are almost devoid of smooth muscle cells (Yoffey and Courne 1971) it seems reasonable to assume that the lymph can easily be filtered through the vascular wall into the tissue. This interpretation is supported by four observations. 1. When no absorption

caused no increase of the plethysmographic volume was noted. 2. When excluding the left end of Fig. 1 from the closed luminal perfusion system a decrease of plethysmographic volume was recorded (Fig. 3), probably reflecting net water absorption of fluid food. The amount of fluid absorbed from the perfusion system was then derived as compliant part, i.e. the intestine, and fluid absorbed via the lymph remained in the lymphograph. 3. Adding together the rate of increase in plethysmographic volume during water absorption, and the rate of decrease recorded after clamping the end of the perfusion system (Fig. 5), one always arrived at a value which was the rate of total net water absorption (Fig. 6). 4. Measurements of lymph flow from intestinal lymph vessels compared well with the volume increase recorded by the lymphograph after clamping the lymph cannula (Table II).

It should be pointed out that the present technique enables not only the study of net water transport but, by addition of proper tracers, bidirectional fluxes of water or any other substance can easily be followed. In such experiments samples are taken of the fluid entering the intestinal segment.

This work was sponsored by grants from the Swedish Medical Research Council (14X 335), from the Greta Johansson stiftelse, from Clara and Lilly Dahlströms fond, from Wilhelm and Martina Thomsénstiftelsen, from Magnus Bergvall's stiftelse and from the Medical Faculty University of Uppsala.

# References

1. R. and F. J. HANSSON, Resorptions- und Sekretionsstudien am Darm. V. Einfluss des osmotischen Drucks auf die Wasserresorption am Duodenum wacher Ratten. *Z. ges. exp. Med.* 1971, 156, 1.
2. O. LINDBERGH and J. WALLINTE, Studies on the relationship between flow resistance, compliance coefficient and regional blood volume in the intestine of the rat. *Acta physiol. scand.* 1963, 78, 283.
3. U. and O. LINDBERGH, Reactions within consecutive vascular sections of the small intestine of during prolonged hypotension. *Acta physiol. scand.* 1972, 84, 151-163.
4. O. LINDBERGH and J. ERIKSSON, On the components of the  $K_{tr}$  wash-out curves from the intestine of the cat. *Acta physiol. scand.* 1968, 72, 257-266.
5. D. S. Methods for investigation of intestinal absorption. I. *Handbook of Physiology* 1966, 14 (Alimentary Canal), vol. III, 1177-1216.
6. M. and F. C. COURTNEY, *L. asphincter, L. ampulla and L. sphincter, cholel ampulla*. Academic Press, London and New York, 1970.

## Acylation of Carnitine and Glycerophosphate in Suspensions of Liver Mitochondria at Varying Levels of Palmitate and Coenzyme

By

B. BORREBAEK

Received 23 May 1975

### Abstract

BORREBAEK, B. *Acylation of carnitine and glycerophosphate in suspensions of rat liver mitochondria at varying levels of palmitate and coenzyme A* Acta physiol. scand. 95 448-456.

Rates of acylation of carnitine and glycerophosphate in suspensions of isolated rat liver mitochondria were measured at varying levels of palmitate and coenzyme A in the presence of ATP. Addition of glycerophosphate caused considerable reduction in carnitine acylation at low but not at high palmitate. Glycerophosphate acylation was less reduced by added carnitine. These results can be explained by lower  $K_m$  (acyl-CoA) in glycerophosphate acylation than in carnitine acylation. High levels of free coenzyme A caused inhibition of carnitine acylation, while the inhibitory effect of glycerophosphate acylation was small. Competition between palmitate and acyl-CoA for binding sites on proteins (e.g. albumin added to the mitochondrial suspensions) was indicated by stimulation of carnitine acylation by palmitate. Palmitoyl-CoA added as substrate in the absence of ATP. Refeeding a carbohydrate-rich diet to previously fasted rats resulted in increased glycerophosphate acylation and decreased carnitine acylation in bovine liver mitochondria.

The rates of oxidation and esterification of fatty acids in the liver are dependent on nutritional state of the animal. A rapid inhibition by glucose of fatty acid oxidation *vice versa* have been demonstrated (Lossow and Chaikoff 1955 Randle *et al.* 1963, Shulman *et al.* 1964), and it has been shown that the glucose effect is correlated with the tissue level of long-chain acylcarnitines (Bohmer 1967 Pearson and Tubbs 1967). This level is at least in part the result of an increased concentration of free fatty acids in blood plasma, leading to increased uptake of fatty acids by the tissues. However, perfused livers from fasted rats oxidize more fatty acids to ketone bodies and carbon dioxide and esterify less to triglycerides than perfused livers from fed animals under identical conditions (Mayes 1970 McGarry *et al.* 1973). It has been shown that the glycerophosphate acylating enzyme (acyl-CoA:sn-glycerol-3-phosphate O-acyltransferase, EC 2.3.1.15) is reduced and that carnitine palmitoyl transferase (acyl-CoA:L-carnitine O-acyltransferase EC 2.3.1.21) is increased in the liver when previously well fed rats are fasted (Norum 1965 Vavrecka *et al.* 1966 Aas and Daae 1971). However, it has not been established that these variations in enzyme

The acylation of carnitine and glycerophosphate by mitochondria from livers of fasted and fasted/refed rats. Rats were refed the carbohydrate diet. Carnitine acylation was performed in the presence of unlabeled glycerophosphate, and glycerophosphate acylation in the presence of unlabeled carnitine. The data, presented together with the S.E. values, represent means of 6-12 observations. The amount of added CoA was 30 nmol. No palmityl-CoA was added.

meal	Mitochondrial protein of the whole liver (g)	Palmitate added ( $\mu$ M)	Carnitine acylation (nmol/min, mg protein)	Glycerophosphate acylation
48 h	$1.07 \pm 0.09$	880	$10.45 \pm 1.21$	$3.19 \pm 0.3$
refed 4 h		220	$2.83 \pm 0.09$	$1.75 \pm 0.16$
48 h	$1.12 \pm 0.11$	880	$9.20 \pm 0.95$	$3.47 \pm 0.27$
refed 16 h		880	$6.40 \pm 0.40$	$6.82 \pm 0.51$
48 h	$1.83 \pm 0.12$	880	$4.72 \pm 0.32$	$5.55 \pm 0.66$
refed 4 h	$1.10 \pm 0.10$	220	$1.82 \pm 0.24$	$3.46 \pm 0.16$

can explain the dietary variations in fatty acid oxidation and esterification. The of acylcarnitines in the liver depends on the rates of fatty acid activation (catalyzed long-chain fatty acid, CoA ligase (AMP-forming) EC 6. 2. 1. 3), glycerophosphate and carnitine acylation. Isolated mitochondria contain the three enzymes catalyzing processes (Farstad *et al.* 1967, Norum and Bremer 1967, Zborowsky and Wojtczak 1968 and Bremer 1970), and represent therefore a suitable and well defined model for a kinetic study of this branching point in fatty acid metabolism.

In the present work, the kinetics of carnitine acylation and glycerophosphate acylation of isolated mitochondria were studied with palmitate, CoA, ATP, carnitine, glycerophosphate as substrates. It is shown that carnitine acylation is inhibited by free  $\text{Ca}^{2+}$  and stimulated by palmitate. It is further shown that increased glycerophosphate and decreased carnitine acylation in the isolated mitochondria were the results of previously fasted rats were refed a carbohydrate diet.

### Methods

Male albino rats of the Wistar strain, weighing 100-200 g were used. The animals were supplied in Møllegaard A. Jensen, Hørsholm, Denmark. They were maintained on stock diet (containing 24% protein, 21% crude carbohydrate, 2.6% fat, 5.6% fiber and 5% ash) obtained from Peter Larsen Co. Oslo, Norway, and free access to water. Fasted rats were deprived of food for 48 h. The fasted/refed rats were fasted for 48 h and then given free access to 20% glucose in the drinking water and were of placebo and crumbled bread for the times indicated. The experiments are performed with mitochondria from rats fed the stock diet, except those presented in Table I.

Materials. The following compounds were obtained from Sigma Chemical Company St. Louis, Mo.: ATP (Ammonium salt, cat. A-3127), DL-glycerophosphate (cat. G-6126), palmitic acid (cat. P-0300), palmityl-CoA (cat. P-1230), Coenzyme A (Lactone salt cat. C-9004),  $\beta$ -NAD (cat. N-7004), acetyl phosphate (cat. A-6003), phosphate acetyltransferase (Acetyl-CoA: orthophosphate acyltransferase EC 2. 3. 1. 8; 1320 U/mg, 5 mg/ml, cat. P-4383), malate dehydrogenase (L. Malate-NAD-dependent EC 1. 1. 1. 37 1300 U/mg, 10 mg/ml, cat. 410-13), and citrate synthase (Citrate oxalo-

## Acylation of Carnitine and Glycerophosphate in Suspensions of Rat Liver Mitochondria at Varying Levels of Palmitate and Coenzyme A

By

B. BORREBAEK

Received 23 May 1975

---

### Abstract

BORREBAEK, B. *Acylation of carnitine and glycerophosphate in suspensions of rat liver mitochondria at varying levels of palmitate and coenzyme A* Acta physiol. scand. 95 448-456.

Rates of acylation of carnitine and glycerophosphate in suspensions of isolated rat liver mitochondria were measured at varying levels of palmitate and coenzyme A in the presence of ATP. Addition of glycerophosphate caused considerable reduction in carnitine acylation at low but not at high palmitate. Glycerophosphate acylation was less reduced by added carnitine. These results can be explained by lower  $K_m$  (acyl-CoA) in glycerophosphate acylation than in carnitine acylation. High levels of free coenzyme A caused inhibition of carnitine acylation, while the inhibitory effect of glycerophosphate acylation was small. Competition between palmitate and acyl CoA for binding sites on protein (cf. albumin) in the mitochondrial suspensions was indicated by stimulation of carnitine acylation by palmitoyl CoA added as substrate in the absence of ATP. Refeeding a carbohydrate rich diet to previously fasted rats resulted in increased glycerophosphate acylation and decreased carnitine acylation in liver mitochondria.

---

The rates of oxidation and esterification of fatty acids in the liver are dependent on the nutritional state of the animal. A rapid inhibition by glucose of fatty acid oxidation *in vivo* has been demonstrated (Lassow and Chalkoff 1955, Randle *et al.* 1963, 1964), and it has been shown that the glucose effect is correlated with the tissue level of long-chain acylcarnitines (Bohmer 1967, Pearson and Tubbs 1967). This level is at least in part the result of an increased concentration of free fatty acids in blood plasma, increased uptake of fatty acids by the tissues. However, perfused livers from fasted rats oxidize more fatty acids to ketone bodies and carbon dioxide and esterify less to triglycerides than perfused livers from fed animals under identical conditions (Mayes 1970, Møller *et al.* 1973). It has been shown that the glycerophosphate acylating enzyme (acyl-sn-glycerol-3-phosphate O-acyltransferase EC 2.3.1.15) is reduced and that carnitine palmitoyl transferase (acyl-CoA: L-carnitine O-acyltransferase EC 2.3.1.21) is increased in the liver when previously well fed rats are fasted (Norum 1965, Vavrocka *et al.* 1965, Aas and Daae 1971). However, it has not been established that these variations in



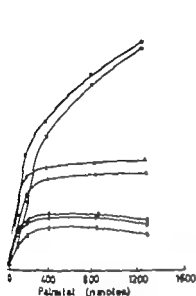


Fig. 1

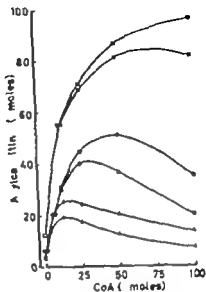


Fig. 2

Carnitine acylation, glycerophosphate acylation, and the level of acyl-CoA at different amounts of substrate. Products are 1: acylcarnitine formed from labelled carnitine in the absence (○) and presence (●) of unlabelled glycerophosphate, 2: acylated glycerophosphate formed from labelled glycerophosphate in the absence (□) and presence (△) of unlabelled carnitine, and 3: acyl-CoA in the presence (○), glycerophosphate (□), or both (●). The amount of added CoA was 50 nmol. No palmitate is added.

Carnitine acylation at different amounts of added CoA. Open symbols indicate the absence and filled symbols the presence of unlabelled glycerophosphate. The amounts of added palmitate were 880 nmol (○, □), 120 nmol (●, △), and 110 nmol (○, △). No palmitoyl-CoA is added.

### Results

Increases in carnitine acylation and glycerophosphate acylation with increasing palmitate levels are shown in Fig. 1. Carnitine acylation was depressed approximately 50% when unlabelled glycerophosphate was added at low palmitate levels. This inhibition ceased when more palmitate was added. Similarly added unlabelled carnitine depressed glycerophosphate acylation, but the effect was relatively small and varied less with the same level. The formation of acylated glycerophosphate was well correlated with the level of acyl-CoA, while production of acylcarnitine still increased with increasing palmitate concentration after the increase in acyl-CoA had ceased (Fig. 1). The results indicate that affinity of glycerophosphate acylating enzyme towards acyl-CoA is higher than that of carnitine acyl transferase.

As evident from Fig. 2 and 3, especially carnitine acylation was inhibited by high levels of CoA. The inhibition decreased with increasing palmitate concentrations. Furthermore, depressing effect of added unlabelled glycerophosphate increased with increasing CoA and decreased with increasing palmitate. The acylation of glycerophosphate was less inhibited at high CoA concentrations. The concentrations of acyl-CoA at different levels of CoA are shown in Fig. 4.

acetyl-CoA lyase (CoA acetylating) EC 4.1.3.7 85 Units/mg, 24 mg/ml, cat. C 3 60). (-) Carnitine (cat. 5991 d) was purchased from Koch Light Laboratories Ltd, Colnbrook, Bucks, England, and Cl reagent (dithiothreitol) from Calbiochem, Los Angeles, Cal. USA. L-3-( $^3\text{H}$ )-glycerophosphate was prepared enzymatically according to Smith & Hubscher (1966), and H methyl labelled (-) carnitine as was the method of Stokke & Bremer (1970). Scintillation liquid was HEC Unisolve I from Koch laboratories.

*Preparation of isolated mitochondria* Rat livers homogenized in 10% sucrose with 1 M (pH 7.4). The homogenate was centrifuged at 800 g for 5 min in order to remove nuclei and cell debris. The mitochondria were sedimented from the supernatant by centrifugation at 8 000 g for 10 min, washed in the same amount of buffer and recentrifuged at 8 000 g for 10 min.

The final pellet was suspended in sucrose solution, so that the concentration of mitochondrial protein in the suspension was 25 mg/ml. All preparative procedures were performed at 0–2°C.

*Incubation procedure* The incubation mixture contained in 1 ml: 100  $\mu\text{l}$  of the mitochondrial suspension (2.5 mg of mitochondrial protein), 0.5 mM TRIS, 5 mM ATP (unless otherwise stated), 2 mM 2 mM KCN, 100 mM KCl, 15 mg/ml of albumin, 1 mM carnitine (unless otherwise stated), 3 mM glycerophosphate (unless otherwise stated), CoA, palmitate and palmityl-CoA as indicated. The pH of the mixture was 7.4. Incubations were carried out at 30°C in glass centrifuge tubes with shaking. Incubation time was 5 min. Carnitine or glycerophosphate in the incubation mixture were labelled by adding small amounts of H labelled compounds with very high specific radioactivity making no changes in the concentrations. Incubations were stopped either by addition of 1 ml butanol and 1 M perchloric acid for 15 s with a whirl mixer or by addition of 100  $\mu\text{l}$  70% perchloric acid. With the exception of the experiments described in Fig. 6 and 7 formation of acylcarnitine and acylated glycerophosphate proceeded linearly during the incubation.

*Assay of acylated carnitine and acylated glycerophosphate* The incubation mixture, shaken with 1 ml butanol, was supplied with 3 ml water, shaken for 15 s with a whirl mixer and centrifuged for 1 min in a table centrifuge. The top layer which contained the acylated glycerophosphate and the acylated carnitine (Dase and Bremer 1970, Bremer 1963), was washed three times with butanol-saturated water. Samples were mixed with 10 ml of scintillation mixture and counted in model 3175 Packard scintillation spectrometer. The amount of acylated carnitine or glycerophosphate was calculated from the measured counts per minute with the specific activity (cpm/amount) of the carnitine or the glycerophosphate in the incubation mixture after subtraction of blank value (cpm at zero time) and background. By this procedure, carnitine acylation was registered when carnitine was labelled and glycerophosphate acylation was measured when glycerophosphate was the labelled compound.

*Assay of long-chain acyl-CoA* Long-chain acyl-CoA was precipitated with the proteins when reactions were stopped by addition of perchloric acid. The precipitate was hydrolyzed with KOH essentially as described by Tubbe & Garland (1964), and the resulting free CoA was estimated by its catalytic effect on combined phosphate acetyltransferase, citrate synthase and malate dehydrogenase. The method is a modification of that reported by Michal and Bergmayer (1970). The assay buffer was 30 ml of 0.2 M buffer containing 100 mg malate, 50 mg NAD, 16 mg acetyl phosphate, 4 mg Cleland reagent (320 Units) malate dehydrogenase, 50  $\mu\text{l}$  (330 Units) phosphate acetyltransferase, and 60  $\mu\text{l}$  (170 Units) citrate synthase at pH 8.0. A sample of 200  $\mu\text{l}$  of the hydrolyzed and neutralized preparation was added to 2 ml of this assay buffer in a cuvette with 10 mm lightpath and the linear increase in optical density at 340 nm and 30°C was registered for 10 min with a Beckman DU spectrophotometer. Measurements with standard preparations of CoA showed that an increase in optical density of 0.5 per 10 min corresponded to about 1 nmol CoA. The results, after subtraction of the value at zero incubation time, were considered to represent the level of long-chain acyl-CoA.

*General* Unless otherwise stated, all data represent the means of 6–8 observations. Some anomalies were observed but the shape of the figures and the variation in the results for different varying parameters were reproducible. Bars indicating statistical variations are therefore included in the figures. The results are consequently presented as nanomoles formed. Since the albumin incubated suspension of mitochondria in all cases was 1 ml, 1 nmol represents 1  $\mu\text{M}$  concentration. It was determined according to Lowry et al. (1951).



Fig. 6

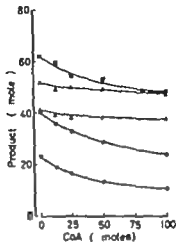


Fig. 7

carnitine acylation and glycerophosphate acylation at different amounts of added palmitoyl-CoA. 1) acylcarnitines formed from labelled carnitine in the absence (○) and presence (●) of 880 nmol, and 2) acylated glycerophosphate formed from labelled glycerophosphate in the absence (△) of 880 nmol added palmitate. The amount of added CoA was 50 nmol. ATP was in the incubation mixture.

inhibitory effect of CoA on acylation and glycerophosphate acylation when palmitoyl-CoA is substrate. Products are acylcarnitines formed from labelled carnitine (● ○, □) and acylated species formed from labelled glycerophosphate (▲ △). Amounts of added palmitoyl-CoA are (●, ▲), 100 nmol (○, □), and 200 nmol (—). The amount of added CoA was 50 nmol. ATP and unlabelled competitive substrates are from the incubation mixture.

The data are not suitable for kinetic analysis, since a significant relative amount of l-CoA disappeared during the incubations, i.e. the time course was not linear so values do not represent initial reaction velocities, especially at low palmitoyl-CoA. Furthermore, since palmitoyl-CoA is partly bound to albumin (Bremer and Norum, 1961), the concentration of free palmitoyl-CoA in the incubation medium is not known. However, the affinity of the glycerophosphate acylating system towards palmitoyl-CoA is higher than that of carnitine acyltransferase. This is in line with data previously obtained by Van Tol (1974).

As is clear from Fig. 7 carnitine acylation was more sensitive than glycerophosphate acylation to inhibition by high levels of CoA. This is in accordance with the results described in Table 1. The degree of inhibition by CoA was highest at the low acyl-CoA level (●, ▲), indicating that the inhibition depends on the CoA/acyl-CoA ratio.

When a carbohydrate diet to previously fasted rats resulted in decreased carnitine acylation and increased glycerophosphate acylation in the isolated mitochondria (Table 1), it is evident that this effect of dietary treatment was not completed after 4 h of refeeding. The rate of mitochondrial glycerophosphate acylation was maximal after 10 h of refeeding (Table 1). This is probably due to a relatively high intake *ad libitum* in the initial refeeding period.

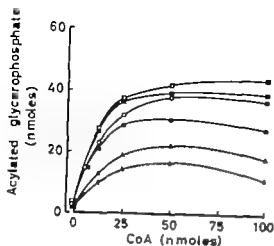


Fig. 3

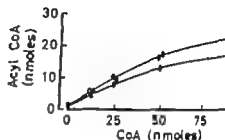


Fig. 4

Fig. 3 Glycerophosphate acylation at different amounts of added CoA. Open symbols indicate the absence and filled symbols the presence of unlabelled carnitine. The amounts of added palmitate are 220  $\mu$ mol ( $\square$ ,  $\blacksquare$ ), 220  $\mu$ mol ( $\circ$ ,  $\bullet$ ) and 110  $\mu$ mol ( $\Delta$ ,  $\blacktriangle$ ). No palmityl-CoA was added.

Fig. 4 The level of acyl-CoA at different amounts of added CoA. The levels of acyl-CoA were from the presence of carnitine ( $\circ$ ), glycerophosphate ( $\bullet$ ) or both ( $\bullet$ ). The amount of added palmitate is 220  $\mu$ mol.

The results with varying concentrations of  $^3$ H labelled carnitine or glycerophosphate at constant specific radioactivities, are described in Fig. 5. The hyperbolic curves correspond to  $K_m(\text{carnitine}) = 0.12$  mM and  $K_m(\text{glycerophosphate}) = 0.31$  mM. The degree of depression by the competitive substrate is about constant at varying concentrations of labelled substrates.

The rates of carnitine acylation and glycerophosphate acylation when varying amounts of palmityl-CoA was added to the incubation system are shown in Fig. 6. In this experiment no ATP was added, so that no synthesis of palmityl-CoA took place. Still, addition of palmitate stimulated carnitine acylation, while there was only little effect on glycerophosphate acylation. Glycerophosphate acylation was inhibited by high concentrations of acyl-CoA.

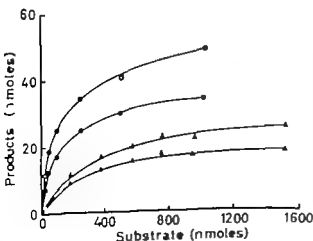


Fig. 5 Carnitine acylation and glycerophosphate acylation at different amounts of added labelled carnitine or labelled glycerophosphate. Products are 1  $\mu$ mol carnitine formed from labelled carnitine in the absence ( $\circ$ ) and presence ( $\bullet$ ) of unlabelled glycerophosphate, and 1  $\mu$ mol acylated glycerophosphate from labelled glycerophosphate in the absence ( $\Delta$ ) and presence ( $\blacktriangle$ ) of unlabelled carnitine. The specific radioactivities of added labelled carnitine and glycerophosphate were kept constant. The amount of added palmitate was 220  $\mu$ mol, and the amount of added CoA was 50  $\mu$ mol. No palmityl-CoA was added.

logical importance. Several reports indicate that this ratio does not change appreciably in rats are fasted (Pearson and Tubbs 1967, Tubbs and Garland 1964, Hoodrop *et al.* 1973, Bortz and Lynen 1963, Greenbaum *et al.* 1971). However, if free fatty acyl-CoA compete for binding sites, a lowering of the fatty acid level due to carbonylation may permit protein binding of acyl-CoA, thus increasing the CoA/acetyl-CoA available to the different enzymes.

The inhibitory effect of glucose on fatty acid oxidation (Lassow and Chaikoff 1955) is mediated through increased insulin secretion, causing decreased levels of fatty acids in the blood and liver tissue. If the data in Fig. 1 are relevant in this context, a fall in level of fatty acids would bring about a relatively proportional decrease in the rate of acylation, and thereby a low level of acylcarnitine. If the tissue level of long-chain acylcarnitines and fatty acid oxidation are correlated with each other as suggested (1967, Pearson and Tubbs 1967), the results would be decreased oxidation of fatty acids, the kinetical characteristics of the system could be a factor in the mechanism glucose-effect. The dietary adaptation of the enzymic reactions is relatively slow (1967), and is therefore not the mechanism of the rapid inhibition of fatty acid oxidation rate, which is established within one hour after glucose feeding (Lassow and Chaikoff 1955, 1967, Jansen *et al.* 1966). However, the dietary adaptation possibly leads to a decrease in fatty acid oxidation after the rapid effect of glucose is established.

This work was aided by grants from Anders Jahre's medical foundation and from Nordic Insulin Fund. Technical assistance of A. Tønnes is gratefully acknowledged. The author is indebted to Professor R. T. Timmer for helpful suggestions and criticism.

## References

- ANDERSON, R. W. DALL, Fatty acid activation and acyl transfer in organs from rats on different nutritional states. *Biochem. biophys. Res. Commun.* 1971, 239, 208-214.
- ANDERSON, R. W. and F. LARSEN, Elevation of long-chain acyl-CoA derivatives in livers of fasted rats. *Biochem. Res. Commun.* 1972, 47, 77-82.
- ANDERSON, R. W., Carnitine in intermediary metabolism, the biosynthesis of palmitoylcarnitine by cell subfractions. *Int. Rev. Cytol.* 1963, 23, 271-279.
- ANDERSON, R. W. and K. R. NORUM, Palmitoyl-CoA: Carnitine O-palmitoyltransferase in the mitochondrial matrix of palmitoyl-CoA. *Eur. J. Biochem.* 1967, 1, 427-433.
- ANDERSON, R. W., Tissue levels of activated fatty acids (acylcarnitines) and the regulation of fatty acids (acylcarnitines) and the regulation of fatty acid metabolism. *Biochem. biophys. Res. Commun.* 1967, 29, 259-264.
- ANDERSON, R. W. and J. REIMER, The acylation of glycerophosphate in rat liver. A new assay procedure for acylglycerophosphate acylation, studies on its subcellular and submitochondrial localization and determination of the reaction products. *Biochem. biophys. Res. Commun.* 1970, 29, 92-104.
- ANDERSON, R. W., J. REIMER and K. R. NORUM, Long-chain acyl-CoA synthase in rat liver. A new assay procedure for the enzyme, and studies on its intracellular localization. *Biochem. biophys. Res. Commun.* 1972, 47, 493-502.
- ANDERSON, R. W., K. A. GUNNA and P. McLEAN, The distribution of hepatic metabolites and the control of the pathways of carbohydrate metabolism in animals of different dietary and hormonal status. *Arch. Biochem. Biophys.* 1971, 141, 617-643.
- ANDERSON, R. W., M. E. ZANETTI and C. F. HUTCHINSON, Studies on lipogenesis *in vivo*. Effects of starvation and re-feeding, and studies on cholesterol synthesis. *Biochem. J.* 1966, 99, 333-340.
- ANDERSON, R. W. and M. GUNNA, The effect of acute and prolonged ethanol treatment on the contents of carnitine, carnitine and their derivatives in rat liver. *Biochem. J.* 1973, 132, 373-379.

## Discussion

It has been suggested that changes in the relative activities of the glycerophosphate acylase and carnitine acylating enzymes depending on diet may regulate the distribution of fat acids between oxidation and esterification in the liver (Aas and Daae 1971). The moderate changes in enzyme activities, and also in the relative high activity of the fatty acid activation enzyme in relation to the acyl-CoA utilizing reactions, gave rather dubious support to the hypothesis. However the results presented here (Table 1) show that the kinetic properties of the enzymes involved combined with changes in the concentration of fatty acids may very well lead to pronounced changes in the fatty acid distribution. Thus, the rate of carnitine acylation at 880  $\mu$ M palmitate in mitochondria from fasted rats was 5-6 times higher than that at 220  $\mu$ M palmitate in mitochondria from fasted/refed rats (Table 1).

It may be objected that only isolated mitochondria have been used in this study while in the intact cell fatty acid activation and glycerophosphate acylation take place both in the endoplasmic reticulum and in the mitochondria (Kornberg and Pricer 1953, Farstad *et al* 1967, Daae and Bremer 1970). However with a more complicated system, e.g. the complete particulate fraction of a liver homogenate, it proved much more difficult to obtain reproducible results. We have therefore performed these primary studies with isolated mitochondria which gave reproducible results. Rat liver mitochondria also contain an appreciable fraction of the total capacities for fatty acid activation and glycerophosphate acylation in that tissue (Farstad *et al* 1967, Daae and Bremer 1970). Isolated mitochondria therefore presumably represent a good model system for these studies.

The small response of glycerophosphate acylation to variations in palmitate concentrations (Fig. 1 and Fig. 6) is most likely explained by a relatively high affinity of the glycerophosphate acyltransferase towards acyl-CoA. It has been previously reported that the  $K_m$  (acyl-CoA) of carnitine acyltransferase is higher than that of glycerophosphate acyltransferase (Van Tool 1974). Also the data presented in Fig. 1 and Fig. 6 indicate a difference in the apparent  $K_m$  (acyl-CoA) values of the two enzymes. It should be stressed that the difference is observed under identical conditions, and is therefore valid even if it is close to meaningless to measure  $K_m$  for a lipid substrate with low solubility and a tendency of binding to proteins and other lipids.

The difference in affinity for the relevant enzymes may be of importance when seen in relation to the binding of acyl-CoA to albumine and presumably other proteins. The stimulation of acylcarnitine formation by palmitate (Fig. 6) is probably due to competition with acyl-CoA for binding sites on proteins, resulting in increased free acyl-CoA and thereby increased acylcarnitine formation. Competition between palmitate and acyl-CoA will lead to a more complete binding of acyl-CoA at low levels of the fatty acid and make carnitine acylation more susceptible towards the competitive action of glycerophosphate (Fig. 1). Similar kinetics as those presented here may be valid in the intact liver cell if some cytoplasmic or structural protein binds fatty acids and acyl-CoA. A fatty acid binding protein has been isolated from animal tissues (Mishkin *et al* 1972, Mishkin and Turcotte 1974).

The results presented in Fig. 7 show that carnitine acylation is inhibited more by free CoA (a high CoA/acyl-CoA ratio) than is the acylation of glycerophosphate. This may be

## Thermoregulation during Static Work with the Legs

By

BOOGE NILSEN

Received 30 May 1975

### Abstract

NI, B. Thermoregulation during static work with the legs. *Acta physiol. scand.* 1975 95: 457-462.

Static work with the legs, i.e. holding a weight of about 10% of maximal isometric strength for 1 h was compared to dynamic exercise on a bicycle ergometer causing the same rate of heat production. In static work the subjective feeling of exertion was very high and the effort maximal. The pronounced increase in heart rate and blood pressure and a typical flush of the face and chest (flush areas) indicated increased sympathetic tones. Plasma catecholamine levels were 1.5 times higher (significant at the  $P < 0.05$ ) after static work than after dynamic work. Although the sustained static work was nearly maximal, the changes in sweating and the change in core temperature during work were not different from those in dynamic work.

Static work deep body temperature is increased in proportion to the relative load on the subject (% of max  $\dot{V}_{O_2}$ ) and proportional to the subjective feeling of exertion. In contrast, static effort, i.e. sustained isometric muscle contraction, is felt very exhausting, and heart rate and blood pressure reach high values, in spite of the low levels of oxygen uptake sustained during this activity (Lund *et al.* 1964). This may be explained as resulting from increased sympathetic tones.

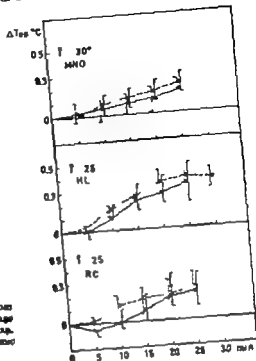
It has been reported that sweat glands are sensitive to adrenaline and noradrenaline (Allen and Roddie 1972). Further Robertshaw and others (1973) demonstrated that restrained macaques (*Macaca species*) sweating during treadmill exercise was related to the increased secretion of adrenaline associated with exercise. In the present study the aim was to compare static and dynamic work to see if the much higher subjective feeling of effort in static work had a specific effect on the thermoregulatory reactions to work.

### Methods

Subjects were three well trained students. Their data are presented in Table 1. For static work the subject sat on a low back-rest with his legs slightly bent (120° between upper and lower leg). He held his feet against a transverse bar supplied with footrests and lifted the suspended weights through his arms. He held the load in a fixed position as long as possible but at least 20 min. For dynamic work he pedalled a Krieger bicycle ergometer in which the saddle was replaced by an armrest. In this way the posture was nearly the same as the two kinds of work.

- LOXOW W J and I L CHAIKOFF, Carbohydrate sparing of fatty acid oxidation. *Arch. Biochem.* 1957 23-40.
- LOWRY O H N J ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the phenyl reagent. *J. biol. Chem.* 1951 193 265-275.
- MAYER, P. A. Studies on the major pathways of lipid metabolism using the perfused liver. In: *Lipid tissue regulation and metabolic functions* Ed. B. Jeanraud and D. Hepp. Georg Thieme Verlag, Stuttgart 1970. 186-195.
- MCGARRY J D J M. MEIER and D W FOSTER, The effects of starvation and refeeding on carbohydrate and lipid metabolism *in vivo* and in the perfused rat liver. The relationship between fatty acid oxidation and esterification in the regulation of ketogenesis. *J. biol. Chem.* 1973 248 270-278.
- MICHAEL, G and H U BERGMAYER, Bestimmung von CoA SH mit phosphotransacetylase. In: *Methoden der enzymatischen Analyse Band II* Ed. H U Bergmayer Verlag Chemie Weinheim/Bergstr 1970. 189-1905.
- MIHAIKIN, S. and R. TURCOTTE, The binding of long chain fatty acid CoA to Z, a cytoplasmic protein present in liver and other tissues of the rat. *Biochem. biophys. Res. Commun.* 1974 57 918-923.
- MIHAIKIN, S., L. STERN, Z. OATMAITAN and J. M. ARIAS, The binding of fatty acids to cytoplasmic protein. Binding to Z protein in liver and other tissues of the rat. *Biochem. biophys. Res. Commun.* 1972 49 997-1003.
- NORUM, K. R. Activation of palmitoyl CoA: carnitine palmitoyltransferase in livers from fasted, lean or diabetic rats. *Biochim. biophys. Acta (Amst.)* 1965 98 652-654.
- NORUM, K. R. and J. BREMER, The localization of acyl-coenzyme A-carnitine acyltransferase in rat cells. *J. biol. Chem.* 1967 242 407-411.
- PEARSON, D J and P K. TUBBS, Carnitine and derivatives in rat tissues. *Biochem. J.* 1967 103 953-9.
- RANDLE, P J C. N. HALES, P. B. GARLAND and E. A. NEWBOLME, The glucose fatty-acid cycle, its role in insulin sensitivity and the metabolic disturbances of diabetic mellitus. *Lancet* 1963 1 785-782.
- STUFF, J C., Interrelation between carbohydrate and fatty acid metabolism of isolated perfused rat liver. *Metabolism* 1964 13 852-860.
- SMITH, M. E. and G. HIRSCHER, The biosynthesis of glycerides by mitochondria from rat liver: the requirement for a soluble protein. *Biochem. J.* 1966 101 308-316.
- STORKE, O and J. BREMER, A simple method for preparation of methyl-labelled ( ) carnitine. *Biochim. biophys. Acta (Amst.)* 1970 218 332-334.
- TUBBS, P. K. and P. B. GARLAND, Variations in tissue contents of coenzyme-A thio esters and possible metabolic implications. *Biochem. J.* 1964 93 350-357.
- VAN TOOL, A. The effect of fasting on the acylation of carnitine and glycerophosphate in rat liver cellular fractions. *Biochim. biophys. Acta (Amst.)* 1974 357 14-23.
- VAVRCKA, M. M. P. MITCHELL and G. HIRSCHER, The effect of starvation on the incorporation of palmitate into glycerides and phospholipids in rat liver homogenates. *Biochem. J.* 1969 115 139-145.
- ZABOLOWSKI, J. and L. WORTZAK, Phospholipid synthesis in rat liver mitochondria. *Biochem. biophys. Acta (Amst.)* 1969 187 73-84.





1. Change in deep esophageal temperature from rest level plotted against work time. Average  $\pm$  SD shown for 3 subjects. Number of exp. Table 1. Static work (SD with range, dynamic) (HL) with range.

## Results

The change in core temperature and the change in weight due to sweating in the first 20-30 min of dynamic and static work are compared in Fig. 1 and 2. For all subjects there is a total of the values, but the time course for weight loss and  $T_{es}$  change appear to be the same in the two kinds of work.

The average values obtained after 20 min of static and dynamic work for  $H$ ,  $V_{O_2}$ ,  $HR$ ,  $T_{es}$ ,  $T_{re}$ ,  $T$  and weight loss are plotted against each other in Fig. 3.

One parameter that is strikingly different in the 2 conditions is the heart rate, which is 4% lower (41%) higher in subject RC, 28 beats (30%) higher in subject HL, and 13 beats (41%) higher in subject MNO during static work.

Table 1

End pressure

Subject	Rest	Dyn	Stat.
	resting	(20 min)	(20 min)
HL	132/92	144/86	153/105
	3	1	-2
RC	128/85	135/91	178/118
	11	5	-6

TABLE I

Subject	Height cm	Weight kg	Max $\dot{V}_{O_2}$	Max HR	Max isom. strength legs kp	Work	
						Dynamic kpm/min	Static kp
HL	189	80	4.45	183	670	360 $n=6$	67 $\pm 6$
RC	180	89	4.71	189	840	450-520 $n=5$	100 $\pm 6$
MNO	186	80	5.19	198	607	198 $n=5$	91-93.6 $\pm 5$

Both work machines could be suspended in a krog balance. Weight loss during the experiments could therefore be measured continuously (Nielsen and Nielsen 1965).

The dynamic work intensity was chosen so as to give the same rate of heat production,  $H$ , as the static work, i.e. oxygen consumption converted to calories,  $M$ , minus the external work in calories,  $W$ , due to dynamic work, equals oxygen consumption (calories) in static work, where no external work is done ( $M = H$ ). This was very nearly obtained as seen in Fig. 3. The higher oxygen uptake during dynamic work is thus explained. The  $\dot{V}_{O_2}$  for the chosen work-load decreased with training in some subjects. It was therefore sometimes necessary to increase the load to keep  $H$  at the initial value.

The respiratory gas exchange was determined by the Douglas bag method. Dried air samples were analyzed on a paramagnetic oxygen analyser (Servomex OA 184) and on an infrared  $\text{CO}_2$  analyser (Beckman LB 1).

Deep esophageal temperature ( $T_{es}$ ) was measured with a thermocouple inserted through the nose and placed in the lower esophagus just above the diaphragm. The placing was controlled in each subject by x-ray photography (Nielsen and Nielsen 1964). The temperature was recorded on a Kipp Micrograph BD 1. The skin temperature ( $T_{sk}$ ) was measured at 15 locations with a thermocouple (Nielsen and Nielsen 1965). The average skin temperature ( $\bar{T}_{sk}$ ) was computed by weighting each measurement according to the size of the corresponding skin area (Hardy and DuBois 1938). In two of the subjects the muscle temperature ( $T_{ms}$ ) was measured with needle probe in the vastus lateralis 2½ cm depth before and after work. The measurements of  $T_{es}$  and  $T_{ms}$  were accurate to within  $\pm 0.5^\circ\text{C}$ ,  $T_{sk}$  to within  $\pm 0.05^\circ\text{C}$ . Heart rate was counted from an ECG registration.

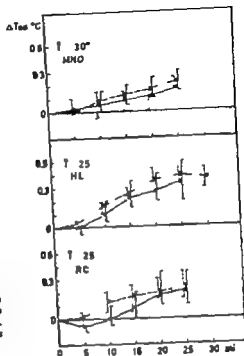
In some experiments on RC, and in all on MNO blood pressure was measured by the auscultatory method over the brachial artery. In HL and RC 5 ml blood sample was taken from the cubital vein 2 min after work had ended and analyzed for lactate by an enzyme method (Scholz *et al.* 1959). In subjects MNO (dynamic or static) work was stopped at exactly 25 min, and 40 ml venous blood from the cubital vein was taken into pre-cooled glass containing 50 mg ascorbic acid and 50 mg EDTA. The blood sample was centrifuged immediately after at 4 000 rev/min for 10 min at  $0^\circ\text{C}$  in cooling centrifuge. The plasma was removed and stored for later analysis for noradrenaline (NA) and adrenaline (A) by the method described by Engelman *et al.* 1968 and 1970.

The experiments took place in a climatic chamber at  $25 \pm 1^\circ\text{C}$  (subj. HL and RC), or  $13.0 \pm 1^\circ\text{C}$  (subj. MNO). Humidity was kept below 75% RH.

### Procedure

The subject arrived fasting. He rested for 45 min in the ergometer seat. The resting  $T_{es}$  and  $\dot{V}_{O_2}$  were recorded and HR counted. Resting metabolic rate was measured in some experiments. Weight loss during rest was registered over a 10-min period.

He then started to work, either pedalling 60 rev/min at low work-load (198-340 kpm/min), giving a rate of heat production equal to that he could maintain for 25 min in static effort — or lifting and holding a weight in the required position for static effort. Heart rate was counted every 5 min.  $T_{sk}$  was measured at approx. 8, 12 and 18 min. Douglas bags were filled at 10 and 16 min. When measured, blood pressure was taken at rest, at 9 and at 19 min work.



Change in deep esophageal temperature from one level plotted against work time. Average values shown for 3 subj. Number of exp. is 1. Static work (○) with range, dynamic (●) with range.

### Results

Change in core temperature and the change in weight due to sweating in the first 20–30 min of dynamic and static work are compared in Fig. 1 and 2. For all subjects there is a rise in the values, but the time course for weight loss and  $T_{es}$  change appear to be the same in the two kinds of work.

The average values obtained after 20 min of static and dynamic work for  $H$ ,  $\dot{V}_{O_2}$ , HR,  $T_{es}$ ,  $T_r$ , and weight loss are plotted against each other in Fig. 3.

The parameter that is strikingly different in the 2 conditions is the heart rate, which is lower (41%) higher in subject RC, 28 beats (30%) higher in subject HL, and 13 beats (24%) higher in subject MNO during static work.

TABLE

and parameters

Subject	Rest	Dyn.	Stat.
MNO	132/72 2	(20 min) 144/86 1	(20 min) 153/105 -2
HL	129/83 11	135/91 3	178/118 -4

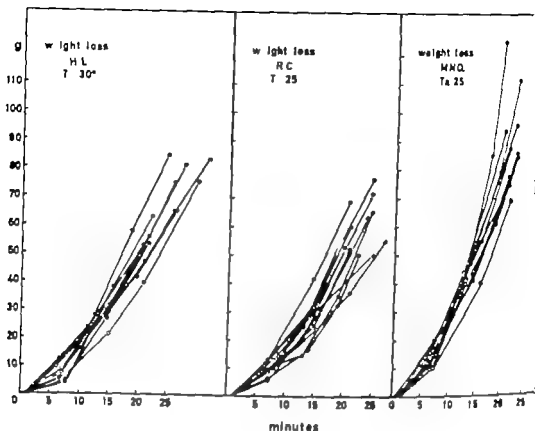


Fig. 2. Total weight loss plotted against work time (3 subj.). Static work ● dynamic work ○

Also blood pressures where measured were higher during static work Table II. Bio lactate after work was 1.6 m mol/l after static, 0.6 m mol/l after dynamic work. When at exhaustion in the static exercise, the subjects showed a very prominent flush of the face at chest ("flush areas")

The plasma catecholamine levels in plasma samples taken after 25 min work were high after static than after dynamic work at the  $P = 0.05$  level. The values are shown in Table II.

### Discussion

The experiments showed that the maximal effort and the very pronounced subjective feeling of exertion during static work had no specific effect on the activation of sweating and the elevation of body temperature.

TABLE III Plasma values g/ml of noradrenaline (NA) and adrenaline (A) after 25 min of static and dynamic work (subj. MNO). Normal values (30). NA  $0.36 \pm 16$ . A  $0.10 \pm 0.03$ .

	NA		A	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.
static work n=6	$0.64 \pm 0.091$		$0.30 \pm 0.050$	
dynamic work n=6	$0.50 \pm 0.050$		$0.23 \pm 0.045$	

Static work values are significantly different from dynamic work values,  $0.01 < P < 0.05$  (T test).

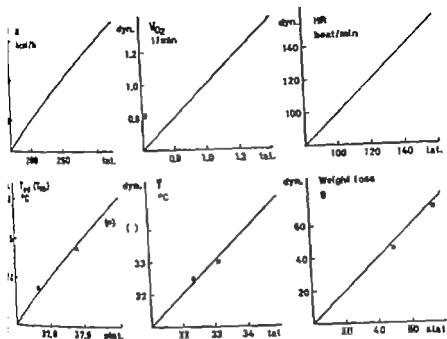


Fig. 3 Comparison of average slope of heat production  $H$ , oxygen consumption  $V_{O_2}$ , heart rate  $HR$ , rectal temperature at 20 min of work  $T_{re}$  (muscle temperature after work brackets ( $T_m$ )), average skin temperature  $T_p$ , and weight loss at 20 min during static and dynamic work. Subj. RC  $\bullet$ , subj. HL  $\circ$ ,  $H^{\circ}C$ .

In prolonged exercise, sweating usually is correlated with rate of heat production (B. Sahlin 1966, 1968, 1969) even when neuromuscular activities are quite different (positive compared to negative work,—arm work to leg work and—as here—static compared to dynamic work). However at the onset of dynamic work (within the first min) the rate of increase in sweating has been found to depend on the intensity of exercise (Beaumont and Mifflin 1963).

The reaction seemed to depend on neural stimuli of central nervous or reflex origin, since a thermal effect on central thermoreceptors could have had time to act.

Koberthaw *et al.* 1973 found that in macaques adrenal denervation decreased sweating during treadmill exercise by approx. 50%, while adrenalin infusion restored sweating to normal levels. It means the sympathetic nerve activity and the plasma level of circulating adrenaline increase during work proportional with the relative load. (Häggendal, Hartley and Sahlin 1970). It is possible that the skin circulation goes down due to the increasing constrictor tone and thus becomes related to the relative load. This again causes the core temperature to increase in proportion to the relative load (Sahlin and Hermansen 1966).

The latter follows from the equation

$$H_{\text{net}} = Q_{\text{sk}} + \dot{V}_{\text{O}_2} \cdot \Delta T_{\text{sk}} + \dot{V}_{\text{O}_2} \cdot \Delta T_{\text{core}}$$

$\dot{Q}$  = skin blood flow

$c$  = heat capacity of blood

}

$\bar{T}_{art}$  and  $\bar{T}_v$  = average temperature of arterial and venous blood from skin.

Kozłowski *et al* (1974) found a significant increase in both NA and A with static (handgrip 30% of max. handgrip) and an increase that was about 1½ times greater than obtained after heavy dynamic work with the legs. In the present static leg work NA twice and A three times higher than values determined by the same method on resting subjects, and both NA and A levels were 1.3 times higher (significant at  $P < 0.05$ ) after static than after dynamic work.

However the present experiments do not support the hypothesis that a high sympathetic tone should force the core temperature upwards. In spite of signs of sympathetic and adrenal activity (high heart rate, blood pressure and higher plasma NA and A) the core temperature during static work increased in the same manner as in the dynamic work, nor did it increase in sweating differ in the two types of work.

I wish to express my gratitude to Dr Frans Fog-Møller, Dept. of Clinical Chemistry, Frederiksberg Hospital, who made the catecholamine analyses.

## References

- ALLEN, J. A. and I. C. RODGER, The role of circulating catecholamines in sweat production in man. *Physiol. (Lond.)* 1972, 27, 801-814.
- BLAUMONT, W. van and R. W. BULLARD, Sweating: its rapid response to muscular work. *Science* 141, 643-646.
- ENGELMANN, K. and D. PORTNOY, A sensitive double-isotope derivative assay for norepinephrine and epinephrine. *Crit. Rev.* 1970, 76, 51-57.
- ENGELMANN, K., B. PORTNOY and W. LOVÉNBERG, Sensitive and specific double-isotope derivative method for determination of catecholamines in biological specimens. *Amer. J. med. Sci.* 1968, 255, 59-66.
- HARDY, J. D. and E. P. DUBOIS, The technique of measuring radiation and conduction. *J. Amer. Physiol.* 1946, 46, 461-475.
- HÄGGGREN, J., L. H. H. RILEY and B. SALTIN, Arterial noradrenaline concentration during even and uneven work: relation to the relative work levels. *Scand. J. Lab. Invest.* 1970, 26, 337-34.
- KOZŁOWSKI, S., Z. BRZEZINSKA, K. N. TAR, W. KOWAL and M. FRANCZYK, Plasma catecholamines during sustained isometric exercise. *Clin. Sci. Molec. Med.* 1973, 45, 723-731.
- LUND, A. R., E. H. TAYLOR, S. H. HUMPHREYS, B. M. KENNEDY and K. W. DONALD, The circulatory response to sustained voluntary muscle contraction. *Clin. Sci.* 1964, 27, 229-44.
- NIELSEN, B., Regulation of Body Temperature and Heat Dissipation at Different Levels of Energy Expenditure. *Acta physiol. scand.* 1966, 68, 215-227.
- NIELSEN, B., Thermoregulatory Responses to Arm Work, Leg Work and Intermittent Leg Work. *Physiol. scand.* 1968, 72, 25-32.
- NIELSEN, B., Thermoregulation at Rest and Exercise. *Acta physiol. scand.* 1969, Suppl. 22.
- NIELSEN, B. and M. NIELSEN, Body temperature during work at different environmental temperatures. *Acta physiol. scand.* 1966, 56, 120-129.
- NIELSEN, B. and M. NIELSEN, On the regulation of sweat secretion in exercise. *Acta physiol. scand.* 1964, 31, 314-322.
- ROBERTSHAW, D. R., TAYLOR and L. H. M. ZIA, Sweat gland secretion by adrenal medulla during exercise. *Amer. J. Physiol.* 1973, 224, 678-681.
- SALTIN, B. and L. HERMANSSEN, Esophageal, rectal and muscle temperature during exercise. *J. Appl. Physiol.* 1966, 21, 1757-1762.
- SCHOLZ, R., H. SCHMITZ, T. BUECHER and J. O. LAMM, Über die Wirkung von N<sub>2</sub> auf die Hämoglobinbildung. *Biochem. Z.* 1959, 331, 71-86.

## Vascular Resistance in Peripheral Blood Vessels at Normotension and at Local Orthostatic Hypertension in Healthy Humans

By

WILLIAM P. PAAKKE and OLE HENRIKSEN

Received 3 June 1975

### Abstract

W. P. and O. HENRIKSEN. *Vascular resistance in peripheral blood vessels at normotension and at local orthostatic hypertension in healthy humans.* Acta physiol. scand. 1975. 95. 463-469.

Effect of changes in orthostatic pressure on vascular resistance in subcutaneous adipose tissue as well as the forearm at normal tension and in the leg at local orthostatic hypertension in three normal subjects. Blood flow in subcutaneous tissue was measured by the local xenon-133 washout technique. Orthostatic pressures are achieved by postural changes of the extremity. At levels above the at which venous pressure is constant, there is autoregulation of blood flow over wider arterial blood pressure ranges in leg than in forearm. When transmural pressure increased 25 mm Hg or more, vascular resistance increased about 50 per cent in the dependent forearm and 300 per cent in the dependent leg. Effect of ischemia on vascular resistance was investigated in cutaneous tissue of hand and foot by use of the  $^{133}\text{Xe}$ -Aulap, new occluder device technique. Vascular resistance after maximum dilatation achieved 20, 30, and 35 mm of ischemia is less in vessels of the hand than in vessels of the foot. Thus, adaptive structural changes of vessels subjected to increased blood pressure are present in cutaneous and subcutaneous adipose tissues in normal subjects.

Hypertensive patients have an increased vasoconstrictor response, augmented constriction for stimulation by noradrenaline, and enhanced resistance after maximum vascular dilatation (Folkow *et al.* 1958, Conway 1963, Shertsov 1970). It has previously been shown that vascular resistance in cutaneous and subcutaneous adipose tissues of the dependent arm increased some 50 per cent compared with resistance at level of the heart (Henriksen *et al.* 1973, Henriksen, Nielsen, and Paaske 1973).

When a person is upright, vessels of the legs are subjected to higher hydrostatic pressures than are vessels of the arm. Thus the effect of changes in orthostatic pressure and vascular resistance during maximum dilatation obtained in areas subjected to local orthostatic hypertension (leg) can be compared to vascular reactions of normotensive areas (arm).

The present study was performed to investigate whether adaptive structural changes of vessels subjected to orthostatic hypertension were present in normal subjects.

## Experimental procedure

The experiments were carried out on three healthy subjects. Room temperature was kept constant at 22°C.

### I Orthostatic pressure changes

(A) *Subcutaneous adipose tissue of forearm.* 0.2–0.4 ml xenon-133 with a concentration of 5 mCi/ml isotonic saline was injected intracutaneously 5 cm proximal to the wrist of the forearm. About 90 min after the injection the xenon-133 is exclusively located in subcutaneous tissue (Sejrsen 1971). This technique used to a small injection trauma to the subcutaneous tissue. After 90 min the measurements are made. The subject was placed in a sitting position and the labelled area was placed at reference level, the jugular notch.

A NaI (TI) scintillation detector was connected to a universal printing gamma-spectrometer (Madsen, Denmark) with an energy window of acceptance adjusted symmetrically around the 0.081 MeV photopeak of xenon-133. A trial consisted of three periods of measurements, each of about 5 min duration, first the labelled area at reference level, then dislocated passively to a test level, and finally again at the reference level.

(B) *Subcutaneous adipose tissue of leg.* Xenon-133 was injected intracutaneously 5 cm proximal to the lateral malleolus. The measurements were performed as described above, the reference level being the iliac crest as the subject was now in supine position.

### II Postischemic hyperemia in cutaneous tissue

Pressures of 80 mm Hg were applied by cuffs placed just proximal to wrists and malleoli of one arm and leg in supine position. 0.05 ml (10–20  $\mu$ Ci)  $^{125}$ I Antipyrine was injected intracutaneously in the heel of each hand and foot 3 cm distal to the cuff. Skin above each deposit was heated by lamps. After 5, 10, 20, 30, and 35 min the cuff were deflated. Washout of  $^{125}$ I was simultaneously recorded by NaI scintillation detectors connected to gamma-spectrometers adjusted symmetrically around the 0.144 MeV photopeak of  $^{125}$ I.

The influence of 35 min of ischemia upon skin temperature was studied separately. Arterial blood pressure was measured with a sphygmomanometer on the arm. Venous blood pressures were measured by venopuncture of a superficial vein of the forearm.

### Calculations

Mean perfusion coefficient,  $\bar{P}$  in subcutaneous adipose tissue was calculated from the Kety equation  $\bar{P} = k \lambda / 100$  (ml/100 g min) where  $k$  denotes the washout rate constant (min<sup>-1</sup>) and  $\lambda$  the blood to adipose tissue partition coefficient (ml/g) (Kety 1951). A  $\lambda$  value of 10 ml/g was used (Sejrsen 1971, Nielsen 1971).

Maximum hyperemic blood flow in cutaneous tissue was estimated from the washout rate constant of the initial monoexponential part of the  $^{125}$ I Antipyrine washout curve. The product of maximum washout rate constant times duration of hyperemia ( $k_{max} t$ ) was calculated (Lindberg 1966, Nielsen and Sejrsen 1972). Arterial mean pressure at reference level ( $\bar{P}_{arm}$ ) was calculated and  $\bar{P}_{arm}$  values are estimated. Venous pressure was measured in forearm and calculated for leg levels. Fractional change of blood flow and relative change in total vascular resistance were calculated for each experiment.

## Results

Results obtained during lowering the extremity are shown in Fig. 1. 1–3 experiments were performed. Relative resistance remained constant when arterial blood pressure was increased from about 90 mm Hg to about 105 mm Hg in both arm and leg. When arterial pressure was further elevated relative resistance increased about 50 per cent in arm and 300 per cent in leg. The difference in vasoconstrictor response was significant.

Results obtained during elevation of the extremity are shown in Fig. 2. 58 experiments were performed. When arterial blood pressure was lowered from 90 mm Hg to about 60 mm Hg (i.e. venous pressure is constant) blood flow remained constant in both arm and leg. Below



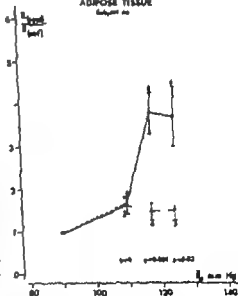
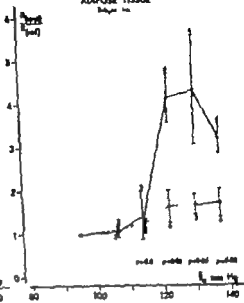
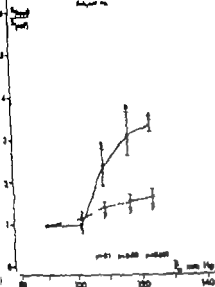


Fig. 1 a, b, and c. Relation change in total vascular resistance ( $R_v/R_0$ ) as function of mean arterial blood pressure ( $P_a$ ). Open circles and the broken line refer to experiments performed on the distal part of forearm, filled circles and the full line to experiments on the distal part of cree. p-values indicate significance level between responses obtained in forearm and cree (rank-sum test for two independent samples). The horizontal bars signify  $\pm S.E.$

at arterial blood pressure of 60 mm Hg blood flow decreased in proportion to the reduction of arterial perfusion pressure in both arm and leg.

Maximum washout rate constants obtained after different duration of ischemia are shown in Fig. 3. 10 experiments were performed on one subject. Maximum washout rate constant did not increase any further when duration of ischemia was extended from 20 min to 35 min.

## Experimental procedure

The experiments were carried out on three healthy subjects. Room temperature was kept constant at 22°C.

### I Orthostatic pressure changes

(A) *Subcutaneous adipose tissue of forearm.* 0.2–0.4 ml xenon-133 with a concentration of 5 mCi/ml isotonic saline was injected intracutaneously 5 cm proximal to the wrist of the forearm. About 90 min after the injection the xenon-133 is exclusively located in subcutaneous tissue (Sejnen 1971). This technique was used to avoid injection trauma to the subcutaneous tissue. After 90 min the measurements were started. The subject was placed in a sitting position and the labelled area was placed at reference level, the jugular notch.

A NaI (Tl) scintillation detector was connected to a universal printing gamma-spectrometer (Mödox, Denmark) with an energy window of acceptance adjusted symmetrically around the 0.081 MeV photopeak of xenon-133. A trial consisted of three periods of measurements, each of about 5 min duration, first with the labelled area at reference level, then dislocated passively to a test level, and finally again with the area at reference level.

(B) *Subcutaneous adipose tissue of leg.* Xenon-133 was injected intracutaneously 5 cm proximal to the lateral malleolus. The measurements were performed as described above, the reference level being the midaxillary line as the subject was now in supine position.

### II Postischemic hyperemia in cutaneous tissue

Pressures of 180 mm Hg were applied by cuffs placed just proximal to the wrist and malleoli of one subject placed in supine position. 0.05 ml (10–20  $\mu$ Ci)  $^{124}$ I Antipyrine was injected intracutaneously in the back of each hand and foot 3 cm distal to the cuffs. Skin above each deposit was heated by lamps. After 5, 10, 20, 30 and 35 min the cuffs were deflated. Washout of  $^{124}$ I was simultaneously recorded by NaI (Tl) scintillation detectors connected to gamma-spectrometers adjusted symmetrically around the 0.664 MeV photopeak of  $^{124}$ I.

The influence of 35 min of ischemia upon skin temperature was studied separately. Arterial blood pressure was measured with a sphygmomanometer on the arm. Venous blood pressures were measured by venopuncture of a superficial vein of the forearm.

### Calculations

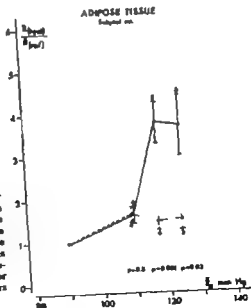
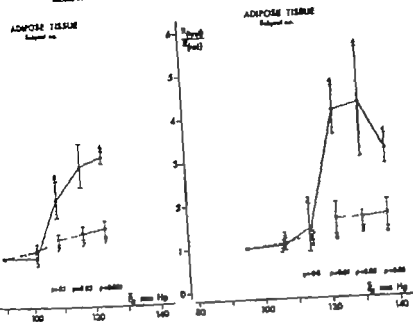
Mean perfusion coefficient,  $\bar{f}$  in subcutaneous adipose tissue was calculated from the Kety equation  $\bar{f} = k \lambda / 100$  (ml/100 g min<sup>-1</sup>) where  $k$  denotes the washout rate constant (min<sup>-1</sup>) and  $\lambda$  the blood to adipose tissue partition coefficient (ml/g) (Kety 1951). A  $\lambda$  value of 10 ml/g was used (Sejnen 1971, Nielsen 1972).

Maximum hyperemic blood flow in cutaneous tissue was estimated from the washout rate constant of the initial monoexponential part of the  $^{124}$ I Antipyrine washout curve. The product of maximum flow rate constant times duration of hyperemia ( $k_{\text{max}} t$ ) was calculated (Lindberg 1966, Nielsen and Sejnen 1972). Arterial mean pressure at reference level ( $\bar{P}_{\text{mean}}$ ) was calculated and  $\bar{P}_{\text{mean}}$  above were calculated. Venous pressure was measured in forearm and calculated for leg levels. Fractional change of blood flow and relative change in total vascular resistance were calculated for each experiment.

## Results

Results obtained during lowering the extremity are shown in Fig. 1. 12 experiments were performed. Relative resistance remained constant when arterial blood pressure was increased from about 90 mm Hg to about 105 mm Hg in both arm and leg. When arterial pressure was further elevated relative resistance increased about 50 per cent in arm and 300 per cent in leg. The difference in vasoconstrictor response was significant.

Results obtained during elevation of the extremity are shown in Fig. 2. 58 experiments were performed. When arterial blood pressure was lowered from 90 mm Hg to about 60 mm Hg (i.e. venous pressure is constant) blood flow remained constant in both arm and leg. Below



## Experimental procedure

The experiments were carried out on three healthy subjects. Room temperature was kept constant at 1

### I Orthostatic pressure changes

(A) *Subcutaneous adipose tissue forearm.* 0.2-0.4 ml xenon-133 with a concentration of 5 mCi/ml isotonic saline was injected intracutaneously 5 cm proximal to the wrist of the forearm. About 90 min after the injection the xenon-133 is exclusively located in subcutaneous tissue (Sejrsen 1971). This technique used to avoid injection trauma to the subcutaneous tissue. After 90 min the measurements are made. The subject was placed in a sitting position and the labelled area was placed at reference level, the jugal notch.

A NaI (Ti) scintillation detector was connected to a universal printing gamma-spectrometer (Medison Denmark) with an energy window of acceptance adjusted symmetrically around the 0.081 MeV photopeak of xenon 133. A trial consisted of three periods of measurements, each of about 5 min duration, first at reference level, then at a level located passively to a test level, and finally again at the reference level.

(B) *Subcutaneous adipose tissue leg.* Xenon-133 was injected intracutaneously 5 cm proximal to the lateral malleolus. The measurements were performed as described above, the reference level being the midaxillary line as the subject was now in supine position.

### II Postischemic hyperemia in cutaneous tissue

Pressures of 280 mm Hg were applied by cuffs placed just proximal to wrists and malleoli of one subject placed in supine position. 0.05 ml (10-20  $\mu$ Ci)  $^{125}$ I Antipyrine was injected intracutaneously in the back of each hand and foot 3 cm distal to the cuffs. Skin above each deposit was heated by lamps. After 5, 10, 15, 20, 30, and 35 min the cuffs were deflated. Washout of  $^{125}$ I was simultaneously recorded by NaI (Ti) scintillation detectors connected to gamma-spectrometers adjusted symmetrically around the 0.364 MeV photopeak of  $^{125}$ I.

The influence of 35 min of ischemia upon skin temperature was studied separately. Arterial blood pressure was measured with a sphygmomanometer on the arm. Venous blood pressures were measured by venopuncture of a superficial vein of the forearm.

### Calculations

Mean perfusion coefficient,  $\bar{f}$ , in subcutaneous adipose tissue was calculated from the Kety equation  $\bar{f} = k \lambda / 100$  (ml/100 g min) where  $k$  denotes the washout rate constant ( $\text{min}^{-1}$ ) and  $\lambda$  the blood/tissue partition coefficient (ml/g) (Kety 1951). A  $\lambda$  value of 10 ml/g was used (Sejrsen 1971, Nielsen 1972).

Maximum hyperemic blood flow in cutaneous tissue was eliminated from the washout rate constant of the initial monoexponential part of the  $^{125}$ I Antipyrine washout curve. The product of maximum washout rate constant times duration of hyperemia ( $k_{\text{max}} t$ ) was calculated (Landbjerg 1966, Nielsen and Sejrsen 1972). Arterial mean pressure at reference level ( $P_{\text{arter}}^{\text{ref}}$ ) was calculated and  $P_{\text{arter}}^{\text{max}}$  values were estimated. Venous pressure was measured in forearm and calculated for leg levels. Fractional change of blood flow and relative change in total vascular resistance were calculated for each experiment.

## Results

Results obtained during lowering the extremity are shown in Fig. 1. 123 expts. were performed. Relative resistance remained constant when arterial blood pressure was increased from about 90 mm Hg to about 105 mm Hg in both arm and leg. When arterial pressure was further elevated relative resistance increased about 50 per cent in arm and 300 per cent in leg. The difference in vasoconstrictor response was significant.

Results obtained during elevation of the extremity are shown in Fig. 2. 58 expts. were performed. When arterial blood pressure was lowered from 90 mm Hg to about 60 mm Hg

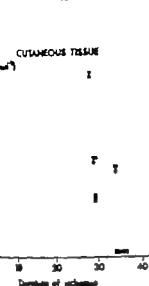


Fig. 3

The maximum washout rate constant ( $k_{max}$ ) of  $^{141}\text{I}$ -Antipyrine washout from cutaneous tissue as a function of duration of ischemia. Open circles denote hand, filled circles foot. Each square represents the mean  $k_{max}$  values between hand and foot in one experiment.

The product of maximum washout rate constant times duration of hypoxemia ( $k_{max} t$ ) as a function of duration of ischemia. Open circles denote hand, filled circles foot. The Spearman rank correlation coefficient,  $r_s$ , 0.99 and 0.91 for hand and foot ( $p < 0.01$ , one-sided significance level, for both regions).

correlation coefficient,  $r_s$ , was 0.99 ( $p < 0.01$ ) for hand and 0.91 ( $p < 0.01$ ) for foot (one-sided significance level).  $k_{max} t$  of hand and foot did not differ (randomization test for paired values,  $p > 0.9$ ).

### Discussion

The effects of orthostatic pressure changes on blood flow in subcutaneous adipose tissue of forearm and leg is qualitatively identical. When the extremity is lowered the vasoconstrictor response to increase in vascular transmural pressure is more pronounced in the leg. The vessels of the leg are subjected to higher transmural pressures the present findings are in agreement with results reported by Folkow *et al.* (1958), Conway (1963), and Sivertsen (1970). During elevation blood flow remains constant confirming that autoregulation of blood flow is present in human subcutaneous adipose tissue (Henriksen, Nielsen, and Sorensen 1973).

$^{141}\text{I}$ -Antipyrine washout is influenced by four components, *i.e.* washout from cutaneous tissue, washout from subcutaneous adipose tissue, transport of tracer from cutaneous to subcutaneous tissue, and trauma of injection.  $k_{max}$  was estimated from the initial slope of the  $^{141}\text{I}$ -Antipyrine washout curve. The interceptions at time zero of the monoexponential tail of the curves obtained in hand and foot accounted for about 10 and 20 per cent of the total height of the washout curve, respectively. This indicates that the initial washout rate

CUTANEOUS TISSUE

 $k_{max} \text{ (min}^{-1} \text{ mm)}$ 

1.0

0.5

0

Duration of ischemia

Fig. 4

## ADIPOSE TISSUE

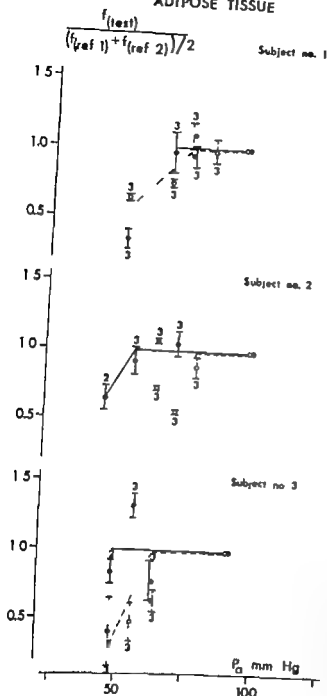


Fig. 2. Relative change of blood flow ( $f_{test}$  ( $(f_{ref 1} + f_{ref 2})/2$ ) as a function of arterial pressure ( $P_a$ ). Open circles and the broken line refer to experiments on the distal part of forearm, filled circles and the full line to experiments on the distal part of crus. The horizontal bars signify 1 S.E.

By comparing  $k_{max}$  obtained from hand and foot after 20, 30 and 35 min of ischemia, the difference in responses was significant at the 95 per cent level ( $p < 0.02$  by randomization test for paired samples). During 35 min of ischemia skin temperature of the areas under study increased about  $1.5^\circ\text{C}$ . Maximum washout rate constant times duration of hyperemia ( $k_{max} t$ ) can be applied in calculating cumulative blood flow ("repayment") (Fig. 4). There was significant correlation between  $k_{max} t$  and duration of ischemia. The Spearman rank



Fig. 3

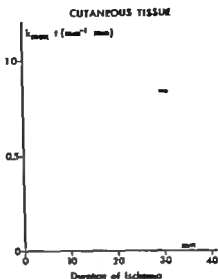


Fig. 4

3. The maximum washout rate constant ( $k_{max}$ ) of  $^{125}\text{I}$ -Antipyrine washed from cutaneous tissue as a function of duration of ischemia. Open circles denote hand, filled circles foot. Each square represents the mean  $k_{max}$  values between hand and foot in one experiment.

4. The product of maximum washout rate constant times duration of hypoxia ( $k_{max} t$ ) as a function of duration of ischemia. Open circles denote hand, filled circles foot. The Spearman rank correlation coefficient,  $r_s$ , 0.99 and 0.91 for hand and foot ( $p < 0.01$ , one-sided significance level, for both regions).

relation coefficient,  $r_s$ , was 0.99 ( $p < 0.01$ ) for hand and 0.91 ( $p < 0.01$ ) for foot (one-sided significance level).  $k_{max} t$  of hand and foot did not differ (randomization test for paired observations,  $p > 0.5$ ).

### Discussion

The influence of orthostatic pressure changes on blood flow in subcutaneous adipose tissue forearm and leg is qualitatively identical. When the extremity is lowered the vasoconstrictor response to increase in vascular transmural pressure is more pronounced in the leg. The vessels of the leg are subjected to higher transmural pressures the present findings are in agreement with results reported by Folkow *et al.* (1958), Conway (1963), and Silvertown (1970). During elevation blood flow remains constant confirming that autoregulation of blood flow is present in human subcutaneous adipose tissue (Henriksen, Nielsen, and Sorensen 1973).

$^{125}\text{I}$ -Antipyrine washout is influenced by four components, viz. washout from cutaneous tissue, washout from subcutaneous adipose tissue, transport of tracer from cutaneous to subcutaneous tissue, and trauma of injection.  $k_{max}$  was estimated from the initial slope of the  $^{125}\text{I}$ -Antipyrine washout curve. The intercepts at time zero of the monoexponential tail end of the curves obtained in hand and foot accounted for about 10 and 20 per cent of the total length of the washout curve, respectively. This indicates that the initial washout rate

from cutaneous tissue is underestimated by less than 10 and 20 per cent in hand and foot respectively. The difference in maximum hyperemic response in hand and foot may be due to different contribution of the washout component for subcutaneous adipose tissue. However, the difference in interception values of about 10 per cent cannot explain the difference in maximum hyperemic response of some 60 per cent. Cutaneous blood flow measured just distal to the cuffs to minimize steal of blood flow from skin to muscle may interfere with the results. It is assumed that the vessels were maximally dilated in both regions as prolonged duration of ischemia did not increase the maximum washout rate considerably any further. As hand and foot were placed at the same level in relation to the heart, arterial perfusion pressures in the two areas are considered to be almost identical.

$k_{max}$  is influenced by the injection trauma. This seems likely from the results presented in Fig. 3 where the washout rate constant obtained in leg after 10 min of ischemia is almost similar to that obtained after 35 min of ischemia. The injection trauma is presumably of the same order of magnitude in all experiments because the traumatically liberated metabolites are not removed until the cuffs are deflated. Therefore, the calculated  $k_{max}$  gives an overestimation of "repayment." No difference in "repayment" was found in the two regions examined ( $p > 0.9$ ).

As cutaneous tissue to blood partition coefficient of  $^3H$ -Antipyrine is about 1 ml/g, blood flow in cutaneous tissue during maximum hyperemia is about 40 ml/100 g/min in hand and about 25 ml/100 g/min in foot. In subcutaneous adipose tissue of the crus maximum blood flow was 15 ml/100 g/min after 30 min of ischemia (Nielsen and Sejrsen 1972). In muscle tissue Lindberg (1966) reported values of 60 ml/100 g/min. The findings indicate that vascular resistance during maximum dilatation is more pronounced in vessels subjected to orthostatic hypertension. Findings by Folkow *et al* (1958, 1971) by Conway (1963), and Sivertsson (1970) are in accordance with this.

Folkow *et al* (1958-1973) ascribe difference in reaction to adaptive structural changes in the wall of vessels subjected to increased transmural pressure, which creates a vascular "hyperreactivity." Results obtained after histometrical examination of the vessels are in accordance with this theory (Furuyama 1961). The findings in the present study indicate that functional changes of vessels subjected to increased blood pressure are present in normotensive subjects. Therefore, the structural changes of vessel walls seem to be a normal physiological adaptation in vessels subjected to increased transmural pressure.

The authors express their gratitude to Dr. Per Sejrsen for advice, discussions, and criticism of the manuscript.

## References

- CONWAY J. A vascular abnormality in hypertension. *Circulation* 1963; 27: 570-579.
- FOLKOW B., G. GRIMBY and O. THULENIUS. Adaptive structural changes of the vascular walls in hypertension and their relation to the control of the peripheral resistance. *Acta physiol scand.* 1958; 44: 227-272.
- FOLKOW B., M. HALLBÄCK, Y. LUNDQVIST, R. SIVERTSSON, and L. WEST. Importance of adaptive changes in vascular design for establishment of hypertension, studied in man and in spontaneously hypertensive rats. *Circulation Res.* 1973; 32: 33, Suppl. 1: 12-13.



- ELIAS, M. Histological investigations of arteries in reference to arterial hypertension. *Tokyo J Med* 1962, 76, 383-414.
- ELIAS, O, S. L. NILSEN and W. P. FARRAR. Autoregulation of blood flow in human adipose tissue. *Scand. J. physiol. med.* 1973, 29, 531-537.
- ELIAS, O, S. L. NILSEN, W. P. FARRAR and P. ESKJER. Autoregulation of blood flow in human adipose tissue. *Acta physiol. scand.* 1973, 29, 533-543.
- FELT, S. Theory and applications of the exchange of inert gas in the lungs and tissues. *Pharmacol. Rev.* 1961, 13, 1-41.
- FRANK, J. F. Leg muscle blood-flow measured with  $^{133}\text{Xe}$  after ischaemia periods and after muscular work performed during ischaemia. *Circ. Sci.* 1966, 30, 399-408.
- LEE, S. L. Adipose tissue blood flow determined by the washout of locally injected  $^{133}\text{Xe}$  mod. *Scand. J. Lab. Invest.* 1972, 29, 31-36.
- LEE, S. L. and P. BUCHTA. Reactor hypertension in subcutaneous adipose tissue in man. *Acta physiol. med.* 1972, 21, 71-77.
- LEE, P. Measurement of cutaneous blood flow by freely diffusible radioactive isotope. Thesis. Copenhagen 1971. *Dansk Medical Bulletin*, 1971, Suppl. 18.
- ROSEN, K. The hemodynamic importance of structural vascular changes in essential hypertension. *Scand. J. physiol. med.* 1970, Suppl. 343.

## Pulsatile Secretion of ACTH, GH, LH and TSH in Man

By

J. LEPPÄLUOTO, U. LAIHA, H. LYBECK, J. PARTANEN, T. RANTA and P. VIRKKUNEN

Received 4 June 1975

---

### Abstract

---

LEPPÄLUOTO J, U LAIHA, H LYBECK, J PARTANEN, T RANTA and P VIRKKUNEN. *Pulsatile secretion of ACTH, GH, LH and TSH in man.* Acta physiol. scand. 1975, 95, 470-476.

Blood samples were taken every 4 min for 80 min from 5 healthy subjects. Plasma ACTH, GH, LH and TSH were estimated by radioimmunoassays. The mean peak intervals were 11, 12, 13 and 14 min, respectively and mean peak amplitudes were 25 pg, 1.1 ng, 11 ng and 5.3  $\mu$ U. Mean durations of ascending and descending limbs of the plasma patterns of these hormones were also measured. The timing of different hormone peaks appeared to be independent.

---

When several blood samples are drawn at short intervals and estimated for hormone concentrations, the blood hormone levels fluctuate continuously. This phenomenon was observed in the secretion of ACTH by Berson and Yalow (1968). Since then experimental evidence has accumulated that other hormones, e.g. cortisol (Hellman *et al.* 1970), (Takahashi *et al.* 1968), FSH and LH (Naftolin *et al.* 1972), LRH (Jeffcoate *et al.* 1974), TSH (Vanhaelest *et al.* 1972) also fluctuate. Instead of remaining at a constant level in plasma, the intermittent increases of the serum hormone levels are called frequent spikes, bursts, peaks or secretion episodes, and are believed to result from simultaneous release of discrete hormone packets from secreting cells into the circulation. Electron microscopic studies on the adenohypophysis support this view because the hormones have been found to be packed in electron-dense granules, which disappear during the secretory phase (Stern *et al.* 1968).

The secretion bursts of adenohypophyseal hormones are evidently initiated by the liberation of releasing hormone, and triggered by the release of neurotransmitters in the hypothalamus. These fluctuations of blood hormone levels have interesting physiological and clinical correlates: the secretion bursts of gonadotrophins develop in adolescence and are amplified after the menopause (Yen *et al.* 1973). In certain amenorrheas the fluctuations are subdued, but in gonadal dysgenesis they are enhanced (Yen *et al.* 1973). Similarly, clomiphene, a drug inducing ovulation, increases the frequency and amplitude of LH secretion bursts (Boyar *et al.* 1973). In previous studies, where samples were taken at 20-60 min intervals, only 6-14 secretion periods per day were found (Boyar *et al.* 1973, Hellman *et al.* 1970).

perimental conditions, however rhythms with a frequency of less than 20 min were detected. When samples were taken more frequently (every 1-3 min) from heifers, a secretion burst of plasma cortisol and GH was observed every 5-10 min (Lam *et al.* 1973).

most results show that the plasma concentrations of ACTH, LH and TSH as of time imply very short intervals between secretion episodes caused by inter-*mittent* of substantial amounts of these hormones into the blood.

### Material and methods

healthy subjects aged 24-32 years (4 males and 1 female) participated in the experiments. They were of the laboratory staff, and four of them (the males) had previously taken part in similar studies (Lam *et al.* 1973).

The experiments were started at 10-11 a.m. A small area above the antecubital vein was washed (Lidocaine® 2%, Orion, Finland, 0.3 ml *s.c.*) and some minutes later cannula (Type AB, Sweden) was inserted into the vein. The subject then sat in a chair in the laboratory for 30 min. 10-ml blood sample was rapidly (maximally in 1 min) withdrawn into plastic syringe 10 U. of heparin per ml. No one felt pain during the venipuncture, but subject No. 5 had vertigo in the beginning of the experiment.

The plasma samples were stored at -20°C for 1-4 months before the estimations. ACTH and GH were assayed with commercial kits (Amersham, U.K. and Kabi, Sweden) and LH with reagents obtained from NIAMDD, Maryland, Bethesda, USA. The sample volume for the assay was 3 ml, for GH 0.1 ml, and for LH and TSH 0.2 ml. Every sample was assayed in duplicate. The assay for GH was done after the extraction procedure. Each hormone was estimated in triplicate except ACTH, which was assayed in four replicates. The antiserum complex formed by the assay was separated on charcoal in the ACTH and GH radioimmunoassays and by antibody method in the LH and TSH radioimmunoassays. The standard curves in the ACTH radioimmunoassays were set up in hormone-free plasma, the others in buffer. The statistical analysis of the RIAs are presented in the following table (Table 1). A significant change in hormone was defined as change of two or more  $\pm$  S.D. The area of  $\pm$  S.D. covers 99.5% of the values.

### Results

The temporal patterns of short-term secretion of ACTH, GH, LH and TSH are shown in Fig. 1 and 2 (subjects 1 and 2). ACTH secretion exhibits two high spikes and other peaks.

The GH level in subject 3 (Fig. 2) was extremely high at the beginning of the experiment but then declined, fluctuating to the normal level. Although this subject complained of vertigo when the cannula was inserted, her ACTH secretion was unchanged in the first 16 min.

LH and TSH displayed from 2 to 4 significant secretion peaks in subjects 1 and 5 in the 80-min experiment (Fig. 1 and 2).

The detailed values for the secretion events in 5 subjects are presented in Table II. In this study each hormone is secreted in 15-19 significant bursts with a mean interval of 15 min. The mean peak amplitude in ACTH secretion was 25 pg, in GH secretion 1.1 ng, in LH secretion 11 ng and in TSH 5.3  $\mu$ U. The respective percentages of the overall mean values are 60, 25, 30 and 90% (Table I). The secretion of GH and especially ACTH

TABLE I.

Hormone	Standard	Intra-assay coefficient of variation	Linear range of the inhibition curve	Normal value	2 S.D. of the normal
ACTH	α 1-4 ACTH	10.5 %	10-400 pg/ml $R = 0.94$	10-75 pg/ml	10 pg/ml
GH	a Kabi standard	3.3 %	1.2-30 ng/ml $R = 0.98$	0.2-8 ng/ml	0.5 ng/ml
LH	LER 907	10.3 %	15-1 000 ng/ml $R = 0.99$	20-36 ng/ml	6 ng/ml
TSH	MRC 63/68	15.5 %	0.75-30 $\mu$ U/ml $R = 0.98$	2-10 $\mu$ U/ml	4 $\mu$ U/ml

<sup>1</sup> Four separate ACTH radioimmunoassays were made: the interassay coefficient of variation was 1.4 %.

<sup>2</sup>  $R$  = regression coefficient, binding percentage (of total) as dependent variable and  $\log_{10}$  hormone concentration as independent variable.  $R$  was always highly significant.

could be divided into low and high peaks, the latter appearing and disappearing rapidly (Fig. 1 and 2 and Table II).

The frequency of coinciding peaks was also recorded, but the timing of the different hormone peaks appeared to be independent. For example, GH secretion coincided with LH peaks 6 times, with TSH peaks 5 times, and with ACTH 4 times, and appeared independently 4 times. When correlations were calculated between every possible pair of plasma hormone levels measured from the same sample for each subject, the only significant correlations were between the LH and TSH levels in two subjects (No. 2 and 3).

The distribution of the hormone concentrations was skewed to the right in every case (Fig. 3), skewness being greatest in the ACTH and GH concentrations. These high ACTH and GH concentrations correspond to the high peaks seen in their secretion.

We regard the "normal" range of values for ACTH as 10-75 pg/ml and during rapid (maximally 1 min) blood sampling there is about a 10% chance of hitting on a high secretion peak, in which the values reach 150-250 pg/ml. In GH secretion our estimates for "normal" values range from 0.2 to 8 ng/ml and there is a similar 10% chance of obtaining GH values up to 40 ng/ml. The two SD ranges for LH and TSH were 20-36 ng/ml and 2-10  $\mu$ U/ml, respectively.

### Discussion

The major findings of the present study can be summarized as follows: 1) Significant peaks in the plasma concentrations of ACTH, GH, LH and FSH were found every 11-14 min (means). 2) The mean peak amplitudes varied from 20 to 160% (maximum even 1 000%) of the pre-peak levels. 3) The duration of the peaks varied from 8 to 14 min. 4) The secretion peaks of the four hormones occurred independently.

The rapid increases and decreases in plasma hormone concentrations imply that each type of adenohypophyseal cell releases discrete packets of its specific hormone intermittently into the blood. Dye dilution studies have shown that a dye takes about 10 s to appear in the blood and that mixing is complete within 6 min (Williams and Deegan 1971). There is no reason to

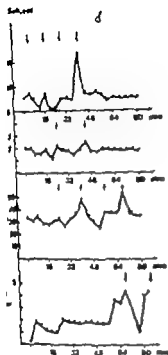


Fig. 1

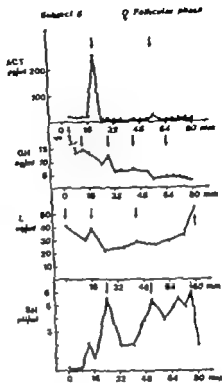


Fig. 2

and 2 T<sup>1</sup> typical temporal patterns of short-term secretion of ACTH, GH, LH and TSH.

that the duration of a hormone burst would differ in any respect during the first few days. In fact, the high secretion peaks of ACTH found in this study conform well to results obtained by the dye dilution technique. The longer duration of certain secretion bursts may be due to our relatively long sampling interval, which would not allow us to detect the peaks and troughs in the hormone duration curve. There is indirect evidence that the secretion bursts of the adenohypophyseal hormones are initiated by the releasing hormones triggered by the hypothalamic neurotransmitters. For example, the plasma LRH concentration in sheep has been demonstrated to indicate brief secretion episodes (Jeffcoats 1974). Although at the moment there is no direct evidence that the secretion of hypothalamic neurotransmitters, releasing hormones and adenohypophyseal hormones are in causal relationship suggested, this appears highly probable. If there were no "external" drivers, the trophic cells would secrete at random and produce a constant plasma level hormone.

ACTH, GH, LH and TSH evidently have different releasing and inhibiting factors and are linked to different hypothalamic neurotransmitter systems, and hence it was expected that secretion peaks of these hormones in the same plasma samples would occur independently. The correlation between the plasma LH and TSH levels found here in two subjects may be due to contamination of LH antiserum with TSH antibodies, as suggested before

TABLE I

Hormone	Standard	Intra assay coefficient of variation	Linear range of the inhibition curve	Normal dose	S.D. of the estimate
ACTH	$\alpha$ 1-4 ACTH	10.5%	10-400 pg/ml R=0.94	10-75 pg/ml	10 pg/ml
GH	a Kabi standard	3.3%	1.2-20 ng/ml R=0.98	0.2-8 ng/ml	0.5 ng/ml
LH	LER 907	10.3	15-1 000 ng/ml R=0.99	20-56 ng/ml	6 ng/ml
TSH	MRC 63/68	15.5	0.78-50 $\mu$ U/ml R=0.98	10 $\mu$ U/ml	4 $\mu$ U/ml

Four separate ACTH radioimmunoassays were made, the interassay coefficient of variation  $\pm 1.4\%$ . R = regression coefficient binding percentage (of total) as dependent variable and  $\log_{10}$  hormone concentration as independent variable. R was always highly significant.

could be divided into low and high peaks, the latter appearing and disappearing rapidly (Fig. 1 and 2 and Table II).

The frequency of coinciding peaks was also recorded, but the timing of the different hormone peaks appeared to be independent. For example, GH secretion coincided with LH peaks 6 times, with TSH peaks 5 times, and with ACTH 4 times, and appeared independently 4 times. When correlations were calculated between every possible pair of plasma hormone levels measured from the same sample for each subject, the only significant correlations were between the LH and TSH levels in two subjects (No. 2 and 3).

The distribution of the hormone concentrations was skewed to the right in every case (Fig. 3), skewness being greatest in the ACTH and GH concentrations. These high ACTH and GH concentrations correspond to the high peaks seen in their secretion.

We regard the "normal" range of values for ACTH as 10-75 pg/ml, and during rapid (maximally 1 min) blood sampling there is about a 10% chance of hitting on a high secretion peak, in which the values reach 150-250 pg/ml. In GH secretion our estimates for "normal" values range from 0.2 to 8 ng/ml and there is a similar 10% chance of obtaining GH values up to 40 ng/ml. The two SD ranges for LH and TSH were 20-56 ng/ml and 10  $\mu$ U/ml, respectively.

### Discussion

The major findings of the present study can be summarized as follows. 1) Significant peaks in the plasma concentrations of ACTH, GH, LH and FSH were found every 11-14 min (means). 2) the mean peak amplitudes varied from 20 to 160% (maximum even 1 000%) of the pre peak levels. 3) The duration of the peaks varied from 8 to 14 min. 4) The secretion peaks of the four hormones occurred independently.

The rapid increases and decreases in plasma hormone concentrations imply that each type of adenohypophyseal cell releases discrete packets of its specific hormone intermittently into the blood. Dye dilution studies have shown that a dye takes about 10 s to appear in the blood and that mixing is complete within 6 min (Williams and Deegan 1971). There is no reason to

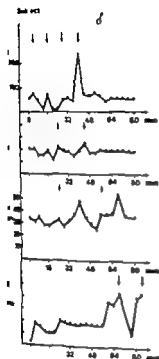


Fig. 1

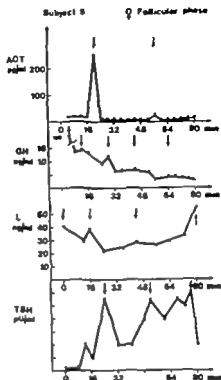


Fig. 2

Fig. 1 and 2. Two typical temporal patterns of short-term secretion of ACTH, GH, LH and TSH.

that the dilation of a hormone burst would differ in any respect during the first few minutes. In fact, the high secretion peaks of ACTH found in this study conform well to those obtained by the dye dilution technique. The longer duration of certain secretion may be due to our relatively long sampling interval, which would not allow us to detect the peaks and troughs in the hormone dilution curve. There is indirect evidence that the bursts of the adenohypophyseal hormones are initiated by the releasing hormones triggered by the hypothalamic neurotransmitters. For example, the plasma LHRH concentration in sheep has been demonstrated to indicate brief secretion episodes (Jeffcoate, 1974). Although at the moment there is no direct evidence that the secretion of hypothalamic neurotransmitters, releasing hormones and adenohypophyseal hormones are in causal relationship suggested, this appears highly probable. If there were no "external" dominants, the tropic cells would secrete at random and produce a constant plasma level instead.

ACTH, GH, LH and TSH evidently have different releasing and inhibiting factors and are part of different hypothalamic neurotransmitter systems, and hence it was expected that the secretion peaks of these hormones in the same plasma samples would occur independently. The correlation between the plasma LH and TSH levels found here in two subjects is probably due to contamination of LH antiserum with TSH antibodies, as suggested before

TABLE II

Hormone peak range	Number of significant peaks	Mean peak interval	Mean peak amplitude	Mean duration of ascending/descending limb
<i>ACTH</i>				
10-100 pg	12	11 min	25 pg	6 min/6 min
over 100 pg	7			4 min/4 min
<i>GH</i>				
0.5- ng	11	12 min	11 ng	5 min/11 min
over 2 ng	8			
<i>LH</i>				
5-20 g	15	13 min	11 g	6 min/11 min
<i>TSH</i>				
4-12 $\mu$ U	18	14 min	5.3 $\mu$ U	8 min/10 min

Total observation time 400 min.

(Aubert and Lemarchand-Beraud 1971) Our calculations on the summated duration of ascending limbs of the secretion peaks indicate that the adenohypophyseal cell secretes during 1/5-1/3 of the time, divided into several episodes per hour. After completion of an adenohypophyseal hormone in the blood circulation its metabolic half-life is

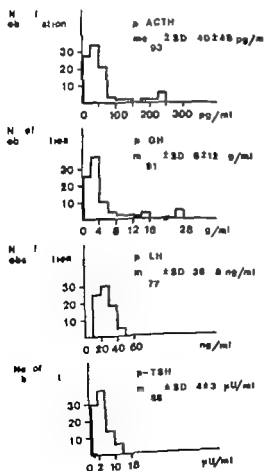


Fig. 3 The distribution of hormone concentrations. The total observation time is 400 min.



shows to vary from 20 to 60 min (Daughaday 1974). The rapid dilution and slow elimination among result is relative rarity of prominent hormone peaks, *i.e.* positive skewness of the distribution of hormone assays. Skewness was greatest in the case of peptide hormones such as ACTH and GH, but did not noticeably disturb the calculation of the  $t$  values for TSH and LH. The ACTH and GH distribution curves were twopeaked, and descends from the high levels representing the hormone-dilution effect. As normal for plasma ACTH and GH we estimate 10–75 pg/ml and 0.2–8 ng/ml, respectively. Take account that the probability of obtaining a single elevated value by chance is 10%. Such a misleading measurement can be avoided by taking several blood samples sample extremely slowly  $\sim 1$  in 10 min.

Obtaining of normal values for GH in this study presented problems. Subject No. 5 initially elevated plasma GH levels at the beginning of the experiment, evidently due to irritation caused by insertion of the cannula. However her ACTH levels were not elevated at this time, showing that ACTH and GH are secreted independently. Subject No. 4 and the 3 subjects had previously participated in a similar experiment and on both occasions had elevated plasma GH levels. In this and the previous study the other subjects had GH levels lower than 4 ng/ml in altogether 115 plasma samples. The height and weight of GH-hypersecretor are normal, as well as his blood sugar level, and he has no clinical evidence of hypersecretion of GH. Hence our normal values for plasma GH are somewhat low but we have no reason to exclude these subjects from our series.

We showed previously that in the resting state, between wakefulness and dozing, the changes in central nervous activity as measured by EEG vigilance, were correlated with bursts of GH secretion, but not with burst of cortisol secretion (Lassi *et al.* 1973). In this study the subjects were awake throughout the experiment and probably at the same EEG vigilance level, but even so had short secretion bursts in their plasma hormone concentrations. It may be that the central nervous system has a permissive effect on the pituitary-gonadotrophic unit.

This work was supported by Grants from the Finnish Medical Foundation and the Fincomb-Nord-svenska Stiftelsen.

## References

- FRANK, M. L. and T. LEMARCHAND-BERAUD, 1971, *Radioimmunoassay methods*. Eds. K. Kirkham and H. H. Edsberg. Churchill Livingstone, 1971. 272–279.
- FRANK, M. L. and R. S. YALOW, 1968, Radioimmunoassay of ACTH in plasma. *J. clin. Invest.* 47, 2725–2731.
- FRANK, M. L., M. FELLOWS, S. KAPLAN, G. LEFKOWITZ, F. WEITZMAN and L. HELLMAN, The effect of clomiphene citrate on the 24-hr LH secretory pattern in normal men. *J. clin. Endocr.* 1973. 36, 561–567.
- FRANK, W. H., 1973, *Textbook of endocrinology*. Ed. R. H. Williams. Philadelphia. W. B. Saunders Co. 1, 11–79.
- FRANK, L., F. NAKADA, J. CLARK, E. D. WEITZMAN, J. KRAM, H. ROYFORD, S. ELLMAN, B. K. FUKUDA and T. F. GALLAGHER, Cortisol is secreted episodically by normal men. *J. clin. Endocr.* 1970. 30, 472–477.
- FRANK, L., H. M. FRANK, D. T. HOLLAND and A. BUNK, Radioimmunoassay of luteinizing hormone-releasing hormone (LH-RH) in serum from men, sheep and rat. *Acta endocr. (Kbh.)* 1974. 75, 635–635.
- FRANK, L., J. LASSI, J. LEPPÄLÖTÖ, J. LUND, J. PÄRKKÄ and P. VIKKUNEN, Growth hormone and prolactin secretion in man. *Acta physiol. scand.* 1973. 89, 193–197.

- NAFTOLIN, F., S. S. C. YEN and C. C. TEAL, Rapid cycling of plasma gonadotrophins in normal men demonstrated by frequent sampling. *Nature* (Lond.) 1972. 236 93-93.
- SZENTAGOTHAL, J. B. FLERKO, B. MESS and B. HALASZ, In *Hypothalamic control of the anterior pituitary*. Budapest Akadémiai Kiadó, 1968. 69.
- TAKAHASHI, Y., D. M. KIPNIS and W. H. DAUGHADAY, Growth hormone secretion during sleep. *J. Invest.* 1968. 47 2079-2090.
- VANHALLET, L., E. VAN CAUTER, J. DEGAUTE and J. GOLSTEIN, Circadian variations of serum thyrotropin levels in man. *J. clin. Endocr.* 1972. 35 479-482.
- WILLIAMS, M. J. and T. DELGAD, <sup>51</sup>Cr-labelled serum albumin in cardiac output and blood flow studies. *Thromb.* 1971. 26 460-465.
- YEN, S. S. C., R. REBAR, G. VANDENBERG and H. JUDD, Hypothalamic amenorrhoea and hypogonadotropism. Responses to synthetic LRF. *J. clin. Endocr.* 1973. 36 811-816.

## Effect of Bulbar Acidification on Basal Secretion of Acid and Gastrin in Dog

By

GÖRAN NILSSON

Received 5 June 1973

### Abstract

NILSSON, G. *Effect of bulbar acidification on basal secretion of acid and gastrin in dog*. Acta physiol. scand. 1975, 95, 477-481.

Dogs prepared with mucosal septal pouches of the stomach and of the duodenal bulb. In some dogs large gastric cannulae was inserted into the most dependent portion of the stomach. In dogs which failed to secrete acid spontaneously during control period at the start of each experiment, the pouches were perfused with 0.1 M HCl for 3-120 min. Bulbar acidification rapidly and profoundly reduced the basal acid output. In dogs which did not secrete acid spontaneously during the control period perfusions were performed with 0.1 M HCl for 1 h. Bulbar acidification did not significantly influence plasma gastrin concentration and no acid was secreted from the Pavlov pouches following such acidification. The present results support the hypothesis that reduction of the intrabulbar pH may contribute to reduction of acid secretion during interdigestive periods. The physiological significance of reduction of the upper intestine which induces acid secretion following reduction of the intraluminal pH is discussed.

Reduction of the intraluminal pH in the duodenal bulb activates a mechanism which is effective in inhibiting gastric acid secretion induced by various stimuli (Andersson and Nilsson 1961, Nilsson 1969 a). The inhibitory mechanism is activated at pHs which normally obtain in the proximal portion of the dog duodenum during postprandial and interdigestive periods. The present study was undertaken to ascertain whether acid perfusion of isolated gastric pouches inhibits spontaneous acid secretion from Pavlov pouches in dog. There is circumstantial evidence that acidification of the mucosa of the upper intestine induces acid secretion (Ivy and McIlvain 1923, Sircus 1953, Code and Watkinson 1955, Sircus 1958). It was therefore decided to investigate whether or not acid perfusion of bulbar pouches influences acid secretion in Pavlov pouch dogs. In addition, the possible influence of bulbar acidification on plasma gastrin levels in dogs was studied.

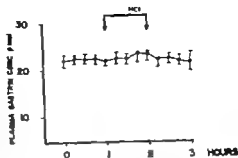
### Methods

#### Surgical procedures

Dogs weighing 12-18 kg were prepared with mucosal septal pouches (Pavlov type) of the stomach and of the proximal portion of the duodenum, corresponding to the duodenal bulb (Andersson and Uvnäs 1961).

- UFTOLIN, F., S. S. C. YEN and C. C. TSAI, Rapid cycling of plasma gonadotrophins in normal men demonstrated by frequent sampling. *Nature (Lond.)* 1972, 236, 92-93.
- VENTA-OOTHAL, J., B. FLERKO, B. MIKES and B. HALASZ, In *Hypothalamic control of the anterior pituitary*. Budapest: Akadémiai Kiadó 1968, 89.
- KAILASHI, Y., D. M. KIPNIS and W. H. DAUGHADAY, Growth hormone secretion during sleep. *J. clin. Invest.* 1968, 47, 2079-2090.
- INHAELST, L., E. VAN CAUTER, J. P. DEGAUTE and J. GOLSTEIN, Circadian variations of serum thyrotropin levels in man. *J. clin. Endocr.* 1972, 35, 479-482.
- WILLIAMS, M. J. and T. DILLON,  $^{51}\text{Cr}$ -Tc labelled serum albumin in cardiac output and blood flow studies. *Thromb.* 1971, 26, 460-465.
- YEN, S. S. C., R. REBAR, O. VANDENBERG and H. JUDD, Hypothalamic amenorrhoea and hypogonadotropinism. Responses to synthetic LRF. *J. clin. Endocr.* 1973, 36, 811-816.

Fig. Plasma gastrin concentrations before, during and after perfusion of isolated bulbar pouches with 0.1 M HCl. Each symbol represents the mean of 9 experiments. Vertical bars represent the range.



**Experimental series.** Following perfusion with acid, the pH of the effluent perfusate varied between 1 and 1.3. Blood samples were collected from jug vein at 15 min intervals. 4-6 blood samples were collected for the determination of basal plasma levels of gastrin. Four samples were taken during the perfusion with acid and another four to six samples after completion of the acid perfusion. The dogs were given large syringes that had been moistened with heparin. Approximately 100 ml of heparin (usually supplied by Virman AB) was added per 4 ml of blood. The blood was then centrifuged immediately and the plasma was removed, frozen and kept at  $-20^{\circ}$  until its gastrin content was determined radioassayably. The method used for the radioimmunoassay of gastrin has been described elsewhere (Nilsson 1975 a).

#### Perfusion series

In both of experiments were undertaken.

**Series 1** was performed on Pavlov pouch dogs whose basal acid output during the control hour was at least 25% of the maximal acid output to histamine. In these dogs the bulbar pouches were perfused with 0.1 M HCl for 5-120 min at a rate of 60 ml per hour.

**Series 2** was undertaken to determine if acid perfusion of bulbar pouches with 0.1 M HCl influenced basal plasma concentrations of gastrin or zodiac acid secretion. The dogs used in this series did not receive any gastrin from their Pavlov pouches during the control hour at the start of the experiment. To avoid any gastric juice that may have been present in the stomach stomach entering the pylorus was and the stomach the gastric contents was kept open during the entire experiment. After control hour of 1 h, the bulb was perfused with 0.1 M HCl for 1 h. Acid secretion and plasma levels of gastrin were followed during the period of bulbar acidification and after stopping the perfusion for another 1 h.

## Results

**Series 1**  
Acid perfusion of bulbar pouches profoundly inhibited the fasting acid secretion from the bulbar pouches. A short period of bulbar acidification was sufficient to evoke inhibition and maintaining a low pH in the bulbar pouches produced a sustained inhibition of acid secretion. These results are illustrated in Fig. 1.

**Series 2**  
Basal plasma gastrin concentrations were not significantly changed following acidification of the bulbar pouches in 9 experiments in 3 dogs (Fig. 2).  
The acid was secreted during the hour preceding the bulbar acidification in these dogs, but the acid perfusion of the bulbar pouches in these or 2 other dogs (in all 15 experiments) did not cause any secretion of acid during either the perfusion or post-perfusion hours. These results are not illustrated.

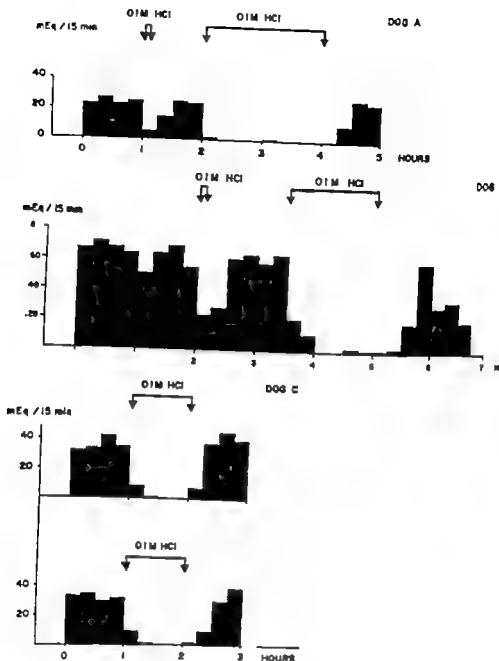


Fig. 1 Basal acid output from Pavlov pouch dogs before, during and after perfusion of bulbar pouch with 0.1 M HCl for 5, 60 and 120 min.

In addition, three of the dogs were provided with a gastric cannula inserted into the most dependent portion of the stomach. Between each operation and before the experimental studies were started the dogs were allowed 3-4 weeks for recovery.

#### Experimental procedures

Experiments were started in the morning after the dogs had been fasted for 18-20 h. Basal acid output was recorded for one hour the secretory responses being collected in 15 min samples. The volume measured and the acidity was determined by titration with 0.01 M NaOH using phenolphthalein as indicator. Perfusion of the bulbar pouches with solutions of 0.1 M HCl was performed as described below.

pH, it is not localized to the bulbar portion of the duodenum. In the postbulbar portion of the dog the intraluminal pH does not reach low levels either during fasting or postprandial periods (Brooks and Grossman 1970). It is therefore questionable if reduction of the intraluminal pH of the postbulbar portion of the intestine under physiological conditions contributes to the stimulation of gastric acid secretion at all.

In summary the present results demonstrate that reduction of the intrabulbar pH probably inhibits spontaneous gastric acid secretion from Pavlov pouches. The results support the concept that acidification of the bulbar mucosa plays a role in inhibiting gastric acid secretion occurring during interdigestive periods. Acid perfusion of bulbar pouches under basal conditions does not induce secretion of acid in Pavlov pouch dogs.

This study was supported by research grants from Statens Medicinska Forskningsråd (project No. 3521) and from Karolinska Institute. The author is grateful to Miss Gunilla Thulin, Miss Elisabeth Tärn and Miss Björnqvist for technical and secretarial assistance.

# References

- BRONKHORST, E. and G. NILSSON, pH-dependence of the mechanism in the duodenal bulb inhibiting gastric acid secretion in conscious gastrin. *Acta physiol. scand.* 1969 70, 182-190.
- BRONKHORST, E. and L. SÖDER, Inhibition of gastric secretion by acidification of the duodenal bulb before and after transection of the pylorus. *Scand. J. Gastroenter.* 1972 7, 151-156.
- BRONKHORST, E. and B. UPPSALA, Inhibition of gastric secretion in Pavlov pouches by instillation of hydrochloric acid into the duodenal bulb. *Gastroenterology* 1961 41, 486-490.
- BRONKHORST, E., G. NILSSON and B. UPPSALA, Effect of acid in proximal and distal duodenal pouches on gastric secretory responses to gastrin and histamine. *Acta physiol. scand.* 1967 71, 368-378.
- BRONKHORST, A. M. and M. L. GROSSMAN, Postprandial pH and neutralizing capacity of the proximal duodenum in dogs. *Gastroenterology* 1970 59, 85-89.
- DAVE, C. P. and G. WATERBURY, Importance of vagal innervation in the regulatory effect of acid in the duodenum on gastric secretion of acid. *J. Physiol. (Lond.)* 1953, 100, 233-252.
- DAVE, A. C. and G. B. MELL, JR., The stimulation of gastric secretion by application of substances to the duodenal and jejunal mucosa. *Amer. J. Physiol.* 1923 67, 126-140.
- ELSTON, O. Studies of the mechanism by which acid in the duodenal bulb inhibits gastric acid secretion. *Physiologia (Stockh.)* 1969 a.
- ELSTON, O., Effect of acid in the duodenal bulb on gastric secretory responses to insulin hypoglycaemia. *Acta physiol. scand.* 1969 b, 73, 476-483.
- ELSTON, O., Effect of acid in the duodenal bulb on gastric secretory responses to sham feeding. *Acta physiol. scand.* 1969 c, 77, 308-315.
- ELSTON, O., Effect of acidification of totally denervated pouches of the duodenal bulb on gastric acid secretion in dogs. *Acta physiol. scand.* 1974, 92, 569-571.
- ELSTON, O., Increased plasma gastrin levels in connection with inhibition of gastric acid responses to sham feeding following bulbar perfusion with acid in dogs. *Scand. J. Gastroenter.* 1975 a, 10, 273-278.
- ELSTON, O., Effect of acid in the duodenal bulb on acid and pepsin responses to test meal in dogs. *Scand. J. Gastroenter.* 1975 b, 10, 279-282.
- ELSTON, O., Effect of bulbar perfusion with acid, hypertonic solutions and acetylcholine on gastric acid secretion in dogs. *Scand. J. Gastroenter.* 1975 c, 10, 283-287.
- ELSTON, O., Effect of bulbar acidification on gastric acid responses to methacholine in Pavlov pouch dogs. *Acta physiol. scand.* 1975 d, 95, 258-262.
- ELSTON, O. and S. RINE, Failure of high PCO<sub>2</sub> in the duodenal bulb to inhibit gastric acid secretion. *Acta physiol. scand.* 1971 81, 349-354.
- ELSTON, O., J. SÖDER, R. S. YALOW and S. A. BRONKHORST, Plasma gastrin and gastric acid responses to sham feeding and feeding in dogs. *Gastroenterology* 1972, 63, 51-59.
- ELSTON, O., The intestinal phase of gastric secretion. *Quart. J. Exp. Physiol.* 1953, 38, 91-99.
- ELSTON, O., Studies on the mechanism in the duodenum inhibiting gastric secretion. *Quart. J. exp. Physiol.* 1954, 39, 114-133.

### Discussion

A series of studies have shown that the mechanism by which acid in the duodenum inhibits gastric acid secretion is mainly localized to the duodenal bulb (Andersson, Nilsson and Uvnäs 1967) and that acid perfusion of bulbar pouches inhibits gastric acid secretion induced by a test meal (Andersson and Uvnäs 1961, Andersson and Sjödin 1972, Nilsson 1975 b) insulin hypoglycemia (Nilsson 1969 b, Andersson and Sjödin 1972) sham feeding (Nilsson 1969 c, Andersson and Sjödin 1972, Nilsson 1975 a) or exogenous gastrin (Andersson, Nilsson and Uvnäs 1967, Andersson and Nilsson 1969, Nilsson and Rune 1971, Nilsson 1974, Nilsson 1975 c and d). Acid responses to histamine (Andersson, Nilsson and Uvnäs 1967) or to the stable choline ester urecholine (Nilsson 1975 d) are not inhibited by the bulbar mechanism.

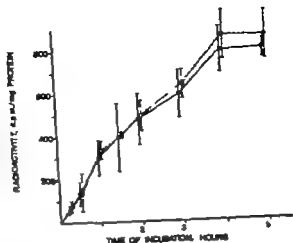
The present study shows that fasting acid secretion is profoundly reduced by acidification of the bulbar mucosa in dogs. As in previous studies (Nilsson 1969 c) using sham feeding as secretory stimulus, a short period of bulbar acidification was sufficient to produce a significant reduction of the basal acid output. Similarly when acidification of the bulbar pouches was prolonged inhibition became more pronounced and was maintained as long as the pH in the bulbar pouch was kept low.

In the present study the intrabulbar pH was reduced to 1–1.3 in all experiments. Other studies show that the bulbar mechanism may be activated at considerably higher pH values. Thus, acid responses to low doses of gastrin (Andersson and Nilsson 1969) or to sham feeding (Nilsson 1969 c) are reduced already at an intrabulbar pH of 3–4. Other experiments in dogs (Brooks and Grossman 1970) show that the pH existing in the fasting duodenal bulb is sufficiently low to activate a pH-sensitive inhibitory mechanism there. Therefore, the present results, together with the studies related above, strongly indicate that a reduction of the intrabulbar pH may play a physiological role in inhibiting interdigestive acid secretion.

The mechanism responsible for the acid secretion which occurs between meals is not known. Experiments in dogs show that atropinization does not significantly reduce the basal plasma gastrin concentration (Nilsson *et al.* 1972). Such observations indicate that basal secretion of gastrin in dog is not under cholinergic control. However, those results do not exclude the possibility that under fasting conditions vagal stimulation may arise in the duodenum causing release of gastrin and cholinergic activation of the parietal cells which in turn results in the secretion of acid. Bulbar acidification liberates a humoral factor (Nilsson 1971) which does not inhibit the release of gastrin but probably competes with gastrin at or close to the parietal cells (Nilsson 1975 a). It seems probable that the inhibition of fasting acid secretion is accomplished in the same way.

In the present study the effect of bulbar acidification on basal gastrin levels in dogs which did not secrete acid spontaneously was also investigated. As found previously (Nilsson 1975 a), perfusion of the bulbar pouches did not influence the basal plasma level of gastrin-like immunoreactivity. Earlier studies (Ivy and McIlvain 1923, Siracus 1953, Code and Wilkinson 1955, Siracus 1958) indicate that perfusion of loops of the upper intestine with strong acid solutions induces secretion of acid from the stomach. Bulbar acidification did not evoke acid secretion from the Pavlov pouches in the present study. If there is a mechanism in the upper intestine of the dog that induces acid secretion following reduction of the intraluminal





Comparison of amino acid incorporation into protein between slices prepared by mechanical chopping and hand. Slices of 80 mg of Hanks medium were incubated with 1.7  $\mu$ M of  $^{14}$ C-L-leucine (activity 34 mCi/mmol) for the indicated times cut by mechanical chopping (O) or by hand (□). The results are means  $\pm$  S.E. of 3 independent experiments each run in duplicate.

for processing and identification. This was achieved by incubating slices under optimal conditions and on a large scale followed by salt fractionation and electrophoretic separation of the proteins.

A preliminary report of this work has been published (Wroński and von der Decken 1973).

### Materials and Methods

**Reagents.** The chemicals used were of per analysis grade wherever possible. DL-leucine- $^{14}$ C (specific activity 34 mCi/mmol, L-valine-2,3- $^3$ H (specific activity 1.5 Ci/mmol and H-leucine specific activity 300 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, Bucks, U.K. L-leucine- $^{14}$ C (U) (specific activity 250 mCi/mmol, L-leucine-4,5- $^3$ H (specific activity 5 Ci/mmol, L-leucine-3,4- $^3$ H (G) (specific activity 100 mCi/mmol from KEN Chemicals GmbH, Frankfurt am Main, Germany) Solucose-300 from Packard Instrument Company, Downers Grove, Ill., U.S.A., Aquasol II from Carlo Erba A.O., Locarno, Switzerland, Leucine 2-15 from Amicon Corp., Lexington, Mass., U.S.A., sterilized Hanks medium from Statens Seruminstitut, Copenhagen, Denmark, glass fiber discs Type A from Gelman Instrument Corp., Ann Arbor, Michigan, U.S.A.

**Animals.** Sprague Dawley male or female rats of 90–130 g or mice of the N.M.R.I. strain of 20 g b.w.

**Brain slices.** The animals were decapitated. The brain was excised, placed in ice-cold medium and sliced. A comparison was made between chopping mechanically using a mechanical chopper similar to that described by M. J. and Biddle (1953), and slicing by hand. As determined with a microtome the approximate thicknesses of 0.50–0.60 and 0.70–0.80 mm for slices cut by mechanical and hand respectively. The incorporation of  $^{14}$ C-leucine into protein was similar for the two preparations (Fig. 1). In the following experiments the mechanical chopper was used.

**Small scale incubations.** This was in Hanks or Krebs Ringer medium the salt composition of which is given in Table I (Hanks and Wallace 1949, Cohen 1951). 80 mg slices in 2 ml were incubated in 25 ml  $^{14}$ C-leucine in 35 ml  $H_2O$ -saturated gas phase of  $O_2$ ,  $CO_2$  (95/5). *(iv)* A mixture of amino acids (Paul 1970) of which was  $^{14}$ C-labelled was added as indicated in the Figures and Tables. After incubation for the time indicated the mixture was transferred to an ice-cold teflon-glass homogenizer and homogenized thoroughly. Samples of 100  $\mu$ l in triplicate were pipetted on to filter paper discs, the proteins were processed (Mann and Novell 1961) and the radioactivity was measured (von der Decken 1968) in an Intertechnique Liquid Scintillation Spectrometer Model SL 30, at 50% efficiency.

To determine the intracellular free  $^{14}$ C-L-leucine after incubation, the slices were centrifuged at 1200 g for 1 min and washed three times with ice-cold medium containing 2000-fold excess of unlabelled leucine in the first washing medium. The slices were then homogenized in 2 ml of medium and 100  $\mu$ l samples were pipetted on to filter paper discs. These are extracted for determination of radioactive proteolysis. T 1 ml

## Synthesis of Brain-Specific Acidic Proteins in Rat and Mouse Cerebral Slices

By

ANDRZEJ WRONSKI AND ALEXANDRA VON DER DECKEN

Received 10 June 1974

### Abstract

WRONSKI A. and A. VON DER DECKEN. *Synthesis of brain-specific acidic proteins in rat and mouse cerebral slices*. Acta physiol. scand. 1975, 95, 482-493.

Optimal conditions for incorporation of radioactive amino acids into proteins by slices of brain have been established. Protein synthesis continued for at least 4 h at 35°C. The highest incorporation of amino acids into protein was obtained with 20-30 mg wet weight per ml incubated at pH 7.1. After incubation of 50% of slices acidic proteins were separated by salifractionation and electrophoresis on 14% polyacrylamide gels. The most acidic band was shown to contain S-100 protein. Identification was solubility of the protein in 100% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, electrophoretic mobility at pH 8.9, precipitation with specific antibodies, and 2-dimensional electrophoresis in polyacrylamide gel. The protein band was insoluble in 50% methanol and contained between 0.06 and 0.2% of the radioactivity present in the total soluble proteins while other cell separated acidic proteins contained about 10 times the radioactivity of S-100 protein.

**Key words.** Brain slices, protein synthesis, S-100 protein, polyacrylamide gel electrophoresis.

It has been reported (Hydén and Lange 1970) that some of the acidic brain proteins may be specifically related to the process of learning. One of these proteins, S-100, has a molecular weight of about 21 000 and contains a relatively high amount of glutamic and aspartic acid (Moore and McGregor 1965). It is easily recognized by its solubility in ammonium sulfate and characteristic electrophoretic migration at a pH higher than 8 (Moore 1965). The amount present in the brain of young adult rats is about 0.2% that of the total protein and the concentration increases in hippocampal nerve cells after training (Hydén and Lange 1970). The apparent sensitivity of S-100 protein towards neurophysiological functions (Zuckerman *et al.* 1970, DeRobertis 1967) would suggest that the protein is also affected by changes in environmental conditions. These would influence primarily the synthetic rate of S-100 and possibly also that of other brain proteins with similar physiological functions.

A necessary step for estimating the synthesis of specific proteins was the establishment of a consistently reproducible system for the *in vitro* incorporation at a high rate of amino acids into protein. In addition the amount of proteins labelled should be sufficient to permit

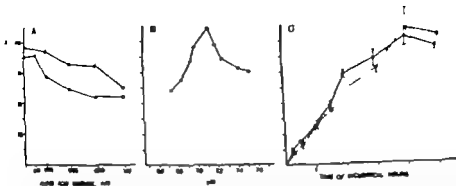


Fig. 2. Conditions for the incorporation of  $^{14}\text{C}$ -leucine into protein by brain slices. Slices of 80 mg in 2 ml of medium were incubated at  $35^\circ$  with  $1.7 \mu\text{M}$  of  $^{14}\text{C}$ -L-leucine (specific activity  $34 \text{ mCi/mmol}$ ). A) Incubation time was for 3 h at increasing concentrations of the amino acid mixture in  $\bullet$ — $\bullet$  Hanks medium and  $\circ$ — $\circ$  Krebs Ringer medium. The results are the means of duplicate incubations. B) pH dependence of the system. Incubation of mouse brain slices in Hanks medium for 3 h in the presence of an amino acid mixture, final concentration  $0.28 \text{ mM}$ . The results are the means of duplicate incubations. C) As in A but the time indicated and with an amino acid mixture, final concentration  $0.28 \text{ mM}$ . The results are the means of 7 independent experiments, each run in duplicate.

## Results

**Characteristics of the incorporation system.** Brain slices from rats or mice were incubated either in Hanks or Krebs Ringer medium. Using the small scale incubation system the conditions for maximal incorporation were studied in detail. The incorporation of  $^{14}\text{C}$ -leucine into protein was enhanced by adding D-glucose to the system showing a broad optimum at  $0.64 \text{ mM}$  and a decrease at higher concentrations of D-glucose. Addition of a mixture of amino acids at low concentrations had no effect on the incorporating activity but increasing amounts inhibited the system (Fig. 2 A). As compared with Hanks medium slices Krebs Ringer medium seemed more sensitive towards the amino acid mixture. The incorporating activity showed a pronounced optimum at pH 7.1 measured at  $35^\circ$  (Fig. 2 B). At incubation of slices for 3 h the pH decreased to 6.95. The incorporation of  $^{14}\text{C}$ -leucine into protein continued for 4 h, and Krebs Ringer and Hanks medium were equally good in supporting protein synthesis *in vitro* (Fig. 2 C). Under the conditions used no significant differences were obtained in the amino acid incorporating activity between slices from rats and mice.

It is known (Ziegler and Melchior 1955) that preincubation of rat liver slices stimulates the rate of amino acid incorporation into protein. Results on the effect of preincubation of brain slices are presented in Table II. A comparison was made between addition of leucine at zero time and at 15 or 30 min preincubation. Radioactivity determined after incubation with the radioactive amino acid present was highest after 15 min preincubation (310 counts) and lowest after 30 min preincubation (230 counts). However at a total preincubation plus incubation time of 90 min the radioactivity values without preincubation (375 counts) and with 15 min preincubation (390 counts) were similar. The uptake of  $^{14}\text{C}$ -leucine was determined by measuring the free intracellular pool of radioactive amino acids (Fig. 3). The maximal uptake was reached after 90 min at  $35^\circ$  while

TABLE I. Composition of the incubation media used.

Chemical	mM concentration of medium	
	Hanks	Krebs Ringer
NaCl	137.00	118.50
KCl	5.35	4.74
MgSO	0.41	1.19
MgCl <sub>2</sub>	0.49	—
CaCl <sub>2</sub>	1.26	—0.09
Na <sub>2</sub> HPO	0.34	—
KH <sub>2</sub> PO	0.44	1.19
D-glucose	53.00	47.00
Phenolred	0.05	0.05
NaHCO	13.90	2.49

of the homogenate. TCA was added to a final concentration of 10%. After centrifugation for 10 min at 15 000  $g$  100  $\mu$ l samples were pipetted on to filter paper discs and the radioactivity of free <sup>14</sup>C-leucine was measured. Free intracellular <sup>14</sup>C-leucine was then calculated after the intercellular plasma of the reticulocyte had been determined with <sup>3</sup>H-ouabain (Regier and Kafatos 1971).

(b) *Large scale incubation.* Slices (300 mg) obtained from brain of 1 or 2 rats or from 2–3 mice were incubated. A 100 ml E-flask containing 10 ml of Hanks medium (Table I), pH 7.1 per 500 mg of tissue was used. To this was added 23  $\mu$ Ci of H-L-amino acid mixture alternatively 23  $\mu$ Ci H-L-leucine and 23  $\mu$ Ci H-L-valine plus the mixture of unlabelled amino acids (Paul 1970) the radioactive ones being omitted (final concentration 0.27 mM) were added. After 5 h incubation at 35°C the slices were homogenized in the incubation medium. Portions of 5 ml were sonicated for 2 min each in an ultrasonic disintegrator 500 Watt Model (M.S.E., London, U.K.) at 1 A and centrifuged for 1 h at 105 000  $g$  (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> III final saturation of 60% (0.390 g/ml) was added to the supernatant under stirring at 4°C. A pH of 8.3 was maintained by adding solid Na<sub>2</sub>CO<sub>3</sub>. After 15 min the suspension was centrifuged for 7 min at 15 000  $g$ . The supernatant was dialysed 14 against 0.15 M NaCl with 4 changes during the first 2 h, followed by 14 h against electrophoresis buffer (see below). The dialysed solution was concentrated with Aquade II or Minicon B 15 at 4°C to 0.5–1 ml and analysed for protein. Radioactivity was determined as described above but instead of filter paper discs glass fibre filters were used. The counting efficiency for tritium was 35%.

*Polyacrylamide gel electrophoresis.* This was essentially as described by David (1964) and Uyemura et al. (1971). The tubes for electrophoresis (120–72 mm) were filled with the resolving 14% gel mixture to a height of 78 mm followed by a layer of 8 mm concentrating gel, pH 7.0 (62 mM Tris-HCl buffer 2.5% w/v, 0.63% butyricamide, 0.5% ammonium persulfate and 0.058% TEMED) 0.58 M sucrose) same concentrating gel but with pH 6.4. After polymerization the test solution (0.6 mg of protein containing traces of bromophenol blue) was layered on top at 3 mA/tube until the dye had reached 1 cm from the bottom of the tube. The buffer consisted of 60 mM Tris-base and 38.6 mM glycine, pH 8.6.

*Radioactivity measurements of the gels.* The gel was cut into 0.33 mm slices. These were placed into one vial. Soluene 350 (0.5 ml) was added, the vial was sealed and shaken for 3 h at 55°C. After cooling 5 ml of Bray's solution (Bray 1960) was added. The vial was sealed and shaken for 3 h at 55°C. The vial was then counted in a liquid scintillation spectrometer at 35% efficiency. A gel run in parallel was stained with Coomassie Brilliant Blue G250 to reveal the protein pattern measured in a Joyce-Loebel Microdensitometer.

*Two-dimensional electrophoresis.* After electrophoresis as described by Hulin and Sjogvist (1971) but

*Immunological identification of S-100 protein.* After polyacrylamide gel electrophoresis the area corresponding to the front band was excised. The protein was recovered by electroelution. The protein was recovered by electroelution. The protein was recovered by electroelution. The protein was recovered by electroelution.

*Analysis.* Proteins were determined by the method of Lowry (1956). Radioactive leucine was identified by 2D gel electrophoresis which was developed in n-butanol/acetic acid.

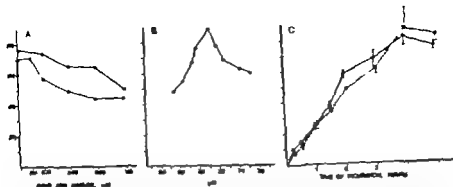


Fig. 2. Conditions for the incorporation of  $^{14}\text{C}$ -leucine into protein by brain slices. Slices of 80 mg in 2 ml were incubated at  $35^\circ$  with  $1.7 \mu\text{M}$  of  $^{14}\text{C}$ -L-leucine (specific activity  $34 \text{ mCi/mmol}$ ). A) Incubation of brain slices for 3 h at increasing concentration of the amino acid mixture in  $\circ$ — $\circ$  Hanks medium and  $\square$ — $\square$  Krebs-Ringer medium. The results are the mean of duplicate incubations. B) pH dependence of *in vitro* incubation of brain slices in Hanks medium for 5 h in the presence of an amino acid mixture, final concentration  $0.23 \text{ mM}$ . The results are the mean of duplicate incubations. C) As in A for the time indicated and with an amino acid mixture, final concentration  $0.23 \text{ mM}$ . The results are  $\pm 3 \text{ S.E.}$  of 7 independent experiments, each run in duplicate.

## Results

**Characteristics of the incorporation system.** Brain slices from rats or mice were incubated either in Hanks or Krebs-Ringer medium. Using the small scale incubation system the conditions for maximal incorporation were studied in detail. The incorporation of  $^{14}\text{C}$ -leucine into protein was enhanced by adding D-glucose to the system showing a broad optimum at 7 to 61 mM and a decrease at higher concentrations of D-glucose. Addition of a mixture of amino acids at low concentrations had no effect on the incorporating activity but increasing amounts inhibited the system (Fig. 2A). As compared with Hanks medium slices a Krebs-Ringer medium seemed more sensitive towards the amino acid mixture. The incorporating activity showed a pronounced optimum at pH 7.1 measured at  $35^\circ$  (Fig. 2B). After incubation of slices for 5 h the pH decreased to 6.95. The incorporation of  $^{14}\text{C}$ -leucine into protein continued for 4 h, and Krebs-Ringer and Hanks medium were equally good in supporting protein synthesis *in vitro* (Fig. 2C). Under the conditions used no significant differences were obtained in the amino acid incorporating activity between slices from rats and mice.

It is known (Ziegler and Melchior 1955) that preincubation of rat liver slices stimulates the rate of amino acid incorporation into protein. Results on the effect of preincubation of brain slices are presented in Table II. A comparison was made between addition of  $^{14}\text{C}$ -leucine at zero time and at 15 or 30 min preincubation. Radioactivity determined after 90 min incubation with the radioactive amino acid present was highest after 15 min preincubation (310 counts) and lowest after 30 min preincubation (230 counts). However at a total preincubation plus incubation time of 90 min the radioactivity values without preincubation (375 counts) and with 15 min preincubation (390 counts) were similar.

The uptake of  $^{14}\text{C}$ -leucine was determined by measuring the free intracellular pool of radioactive amino acids (Fig. 3). The maximal uptake was reached after 90 min at  $35^\circ$  while

TABLE 1 Composition of the incubation media used.

Chemical	mM concentration of medium	
	Hanks	Krebs Ringer
NaCl	137.00	118.50
KCl	5.35	4.74
MgSO <sub>4</sub>	0.41	1.19
MgCl <sub>2</sub>	0.49	—
CaCl <sub>2</sub>	1.26	2.09
Na <sub>2</sub> HPO <sub>4</sub>	0.34	—
KH <sub>2</sub> PO <sub>4</sub>	0.44	1.19
D-glucose	53.00	47.00
Phenolred	0.05	0.05
NaHCO <sub>3</sub>	13.90	2.49

of the homogenate TCA was added to a final concentration of 10%. After centrifugation for 10 min at 15 000  $g$  100  $\mu$ l samples were pipetted on to filter paper discs and the radioactivity of free  $^3\text{H}$ -leucine was measured. Free intracellular  $^3\text{H}$ -leucine was then calculated after the intercellular volume of the weight had been determined with  $^3\text{H}$ -inulin (Reger and Kafatos 1971).

(b) *Large scale incubation.* Slices (500 mg) obtained from brain of 1 or 3 rats or from 2–3 mice incubated. A 100 ml E flask contains 90 ml of Hanks medium (Table 1), pH 7.1 per 500 mg of slices was used. This was added 23  $\mu$ Ci of  $^3\text{H}$ -L-amino acid mixture alternatively 23  $\mu$ Ci  $^3\text{H}$ -L-leucine 23  $\mu$ Ci  $^3\text{H}$ -L-valine plus the mixture of unlabelled amino acids (Paul 1970) (the radioactivity being 0.05 (final concentration 0.27 mM)) were added. After 5 h incubation at 35° the slices were homogenized in incubation medium. Portions of 5 ml were sonicated for 2 min each in an ultrasonic disintegrator 500 V Model (M.S.E. London, U.K.) at 1 A and centrifuged for 1 h at 105 000  $g$  ( $(\text{NH}_4)_2\text{SO}_4$  at a final molar of 60% (0.390 g/ml) was added to the supernatant under stirring at 4°. A pH of 8.3 was maintained adding solid  $\text{K}_2\text{CO}_3$ . After 15 min the suspension was centrifuged for 7 min at 15 000  $g$ . The supernatant was dialyzed at 4° against 0.15 M NaCl with 4 changes during the first 2 h, followed by 14 h against electrophoresis buffer (see below). The dialyzed solution was concentrated with Aquade II or Marcon B at 4° to 0.5–1 ml and analysed for protein. Radioactivity was determined as described but instead filterpaper discs glass fibre filters were used. The counting efficiency for tritium was 35%.

*Polyacrylamide gel electrophoresis.* This was essentially as described by David (1964) and Ujcsanyi (1971). The tubes for electrophoresis (120–72 mm) were filled with the resolving 14% gel mixture to height of 78 mm followed by a layer of 8 mm concentrating gel, pH 7.0 (62 mM Tris-HCl buffer, 2.5% acrylamide, 0.63% bisacrylamide, 0.5% ammonium persulfate and 0.05% TEMED, 0.58 M sucrose) and 8 mm of 1% same concentrating gel but with pH 6.4. After polymerization the test solution in 0.25 M sucrose (0.6 mg of protein containing traces of bromophenol blue) was layered on to the gel. Electrophoresis at 3 mA/tube until the dye had reached 1 cm from the bottom of the tube. The electrophoresis buffer consisted of 60 mM Tris-base and 38.6 mM glycine, pH 8.6.

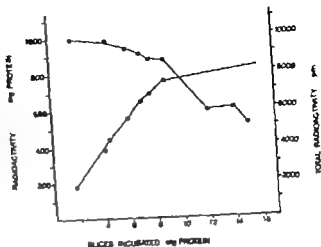
*Radioactivity measurements of the gels.* The gel was cut into 0.33 mm thick slices with a gel-slicer and 51 tubes were placed into one vial. Soluene-350 (0.5 ml) was added, the vial covered with its cap and heated for 3 h at 55°. After cooling 5 ml of Bray's solution (Bray 1960) was added before counting in the scintillation spectrometer at 28% efficiency. A gel run in parallel was stained with Amido black 10 B, destained and the protein pattern measured (Joyce Loeb Microdensitometer Model E 12 MK III).

*Two-dimensional electrophoresis.* After electrophoresis as described but the gels were run in a second direction as described by Hultin and Sjödqvist (1971) but in 14% acrylamide.

*Immunological identification of S-100 protein.* After polyacrylamide gel electrophoresis one gel was stained for proteins. The area corresponding to the front band of the stained gel was excised from the unstained gels run in parallel. The protein was recovered electrophoretically (Kaltchmidt and Wilmann 1969) using a cationic filter. The double-diffusion technique of Ouchterlony (1948) was used to identify the protein immunologically.

*Analyses.* Proteins were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Radioactive leucine was identified by 2-dimensional thin layer chromatography on substage plates which were developed in  $n$ -butanol:acetic acid:  $\text{H}_2\text{O}$  (60:20:20,  $v/v/v$ ) and phenol:  $\text{H}_2\text{O}$  (75:25,  $v/v$ ).

Correlation between slices incubated and incorporated into proteins (as mg protein) were 1 under conditions described in Fig. 2 B.  $\bullet$ — $\bullet$   $^{14}\text{C}$  incorporated per mg  $\text{O}$ — $\text{O}$  total incorporation into proteins. The is the mean of 2 incubations.



free leucine gave a strong positive reaction with ninhydrine where the radioactive  $^{14}\text{C}$  is recovered. The results show that metabolic conversion of  $^{14}\text{C}$ -leucine was neg-

ligible. In experiments described so far the final incubation was 2 ml containing slices of 80 mg wet weight, which corresponds to 7-8 mg of protein. As illustrated in Fig. 4 the specific incorporation of  $^{14}\text{C}$ -leucine per mg protein was fairly constant up to 9 mg of total protein thereafter decreased with increasing concentrations. On the other hand, there was a marked increase in total amount of  $^{14}\text{C}$ -leucine incorporated with increasing amounts of slices which levelled off at 160 mg wet weight (14-16 mg of protein). Thus, with 80 mg of slices present in 2 ml medium, not only a high specific incorporation, but also a high total incorporation was obtained.

*Incorporation of H-amino acids into acidic proteins.* In order to obtain a sufficient amount of protein for isolation of the acidic brain proteins the large scale incubation system was used. Preliminary experiments had shown that it was difficult to elute after electrophoresis the acidic proteins from the 14% polyacrylamide gel and to make them available for liquid scintillation counting at a high yield. A 70% recovery of the radioactivity present in the gels was obtained after treatment of 0.3 mm thick slices with Soluene 350 at 55°.

After incubation of slices with H-amino acids acidic proteins were isolated by  $(\text{NH}_4)_2\text{SO}_4$  fractionation and electrophoresis on polyacrylamide gel. Fig. 5 shows the pattern of the acidic proteins from mice together with the radioactivity distribution. Fig. 5 A shows the radioactivity distribution after extraction of the unstained gel with Soluene 350. Fig. 5 B shows the radioactivity distribution after extraction of the stained gel with Soluene 350. The results show that extraction of radioactivity from the gels after staining and destaining resulted in loss of radioactive proteins which had not been sufficiently fixed by the staining procedure.

The pattern of rat brain proteins and radioactivity distribution are shown in Fig. 6. The 3-100 protein together with other acidic proteins were well separated from the bulk of soluble proteins. In the case of the 3-100 protein containing band the radioactivity amounted to 106-0.2% that of the total soluble proteins.

TABLE II Effect of preincubation of slices on the incorporation of  $^{14}\text{C}$ -leucine into proteins. Rat brain slices were incubated under the conditions described in Fig. 2 B.  $^{14}\text{C}$ -leucine was added at zero time or at 15 or 30 min after the start of incubation. The total incubation time of the slices is indicated 60, 75 or 90 min. The results are the mean of 2 independent experiments.

$^{14}\text{C}$ -leucine added	Radioactivity counts/min/mg protein		
	at 60 min	70 min	90 min
zero time	270	310	375
after 15 min	265	310	390
after 30 min	150	180	230

the synthesis of the intracellular proteins continued to increase for another 150 min. Similar results were obtained with slices from rats and mice. The amount of intracellular radioactive amino acid was calculated after the intercellular space had been determined with H-ex (Rieger and Kafatos 1971). The intercellular space was found to be  $32.7 \pm 1.6 \mu\text{l}$  per 100 of wet weight tissue.

It has been reported (Banker and Cotman 1971) that amino acids are metabolized in brain and thus not utilized as precursor for proteins. Therefore, the amount of free  $^{14}\text{C}$ -leucine present in the slices after 5 hours of incubation was determined by 2-dimensional thin layer chromatography. 95% of the radioactivity applied to the origin was recovered in the band corresponding to the  $R_f$  values of leucine. These are 0.47 and 0.48 for the butanol and phenol system respectively (Randerath 1962). A control run in parallel and containing excess

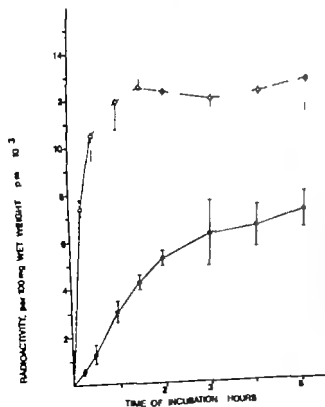
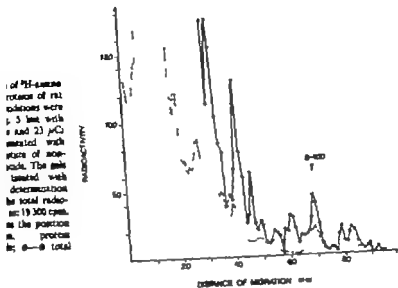


Fig. 3 Uptake of  $^{14}\text{C}$ -L-leucine by brain slices. Slices were incubated  $0.23 \mu\text{M}$   $^{14}\text{C}$ -L-leucine (specific activity  $250 \text{ mCi/mmol}$ ) for the time indicated. The amount of  $^{14}\text{C}$ -leucine present in tissue and that incorporated into protein was determined (see Material and Methods). The intercellular space was measured using H-ex and found to be  $32.7 \pm 1.6 \mu\text{l}/100 \text{ mg}$  wet weight. The amount of intracellular free  $^{14}\text{C}$ -leucine was calculated. The results are the mean  $\pm$  S.E. of 3 independent experiments, each run in duplicate. Radioactivity  $100 \text{ mg}$  wet weight, of protein  $\bullet$ — $\bullet$  free intracellular  $^{14}\text{C}$ -leucine  $\circ$ — $\circ$ .





ties of S-100 protein. The band migrating in front contained S-100 protein and with an arrow in Fig. 5 and 6. S-100 protein was identified by its characteristic mobility (Moore 1965, Moore and McGregor 1965). A purified S-100 protein coelectrophoresed with the front band and an increase in intensity was obtained. Another characteristic of S-100 protein is its solubility in 60%  $(\text{NH}_4)_2\text{SO}_4$ . As shown in Fig. 7 the fraction remaining in solution at 60% saturation contained several separated acidic proteins which were almost absent at 100% saturation. Soluble S-100 protein remained in solution at the high salt concentration. The band seen after precipitation at 60% saturation of  $(\text{NH}_4)_2\text{SO}_4$  and which is similar to the S-100 protein is presumably the 14-3-2 protein described by Hydén (1970). This protein fraction was insoluble at 100%  $(\text{NH}_4)_2\text{SO}_4$ . The front band was further identified as containing S-100 protein by precipitation with antibodies to beef S-100 protein (Fig. 8).

It is likely that the S-100 protein of the fraction soluble at 60%  $(\text{NH}_4)_2\text{SO}_4$  separates from other proteins in the one-dimensional polyacrylamide gel electrophoresis. It has been shown (Haglid and Stavrou 1973) that the front band after electrophoresis in 10% polyacrylamide consists of more than a single protein. The additional bands are glycoproteins soluble in 50% methanol (Ramirez *et al.* 1974, Haglid *et al.* 1975). A two-dimensional electrophoresis in polyacrylamide was run to analyse the proteins in the second electrophoresis contained SDS to assure good separation under these conditions. The S-100 protein-containing band of the first electrophoresis resolved into a complex. This is often observed after a one-dimensional electrophoresis in a continuous medium (Haglid and Stavrou 1973). To remove the glycoprotein one gel slab was soaked in ethanol for 4 h before being stained with Amido black (Fig. 9 B). No convincing differences were observed between the treated and untreated gel slabs (Fig. 9 A and B).

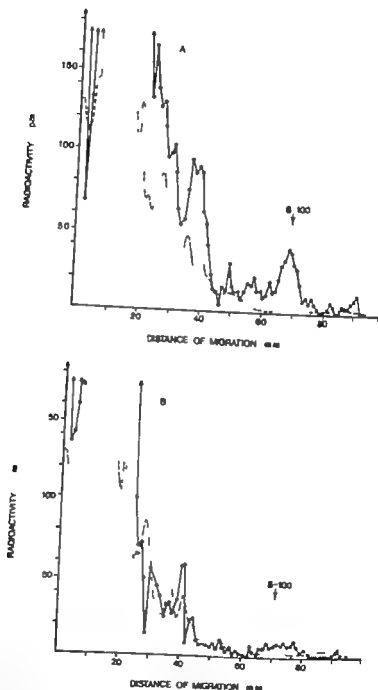


Fig. 5 Incorporation of  $^3\text{H}$ -amino acids into soluble proteins of mice brain. Slices (500 mg) were incubated in 10 ml of Hanks medium with 23  $\mu\text{Ci}$  of the  $^3\text{H}$ -amino acid mixture. After incubation of 5 h at  $35^\circ\text{C}$  the slices were processed, the soluble proteins fractionated, concentrated with Minicon B 15 and subjected to polyacrylamide gel electrophoresis. A) The gels were sliced and treated with Soluene-350 before determination of radioactivity. B) The gels were stained and destained before treatment with Soluene-350 and radioactivity determination. The total radioactivity recovered was A) 17 900 cpm, B) 9 000 cpm. The arrow indicates the position of the S-100 protein. — protein pattern of the gels ●—● total radioactivity



Fig. 2. Two-dimensional electrophoretic pattern of brain proteins soluble at 60%  $(\text{NH}_4)_2\text{SO}_4$ . The arrow indicates S-100 protein. (a) The gel was stained and destained directly after electrophoresis. (b) The gel was stained and destained after 30 min mechanical treatment before staining and destaining.

with isoelectric focusing rather than with conventional disc electrophoresis. Under these conditions the glycoprotein may be retarded differently from that of S-100 protein. The incorporation of amino acids into the S-100 protein containing band is relatively low as compared with the synthesis of serum albumin by rat liver slices (Campbell and Stone 1957). This can be explained partly by the low concentration of this protein present in the brain (Hydén and Lange 1970). The half life of S-100 protein has been reported to be 16 days (Cline and Moore 1970), 3 days (Herichmann 1971) or 1 day and less (McEwen and Hydén 1969). However the low incorporation of radioactive amino acids would indicate a long half-life.

The radioactivity incorporated into the band containing S-100 protein was between 0.06 and 0.1% that of the total soluble proteins. This is consistent with the findings by other workers *in vivo* (0.1–0.5%, Zornitzky-Neurath *et al.* 1972) and in cultures of human glial cells (0.06%, Herichmann 1971).

The incorporation of radioactive amino acids into acidic proteins other than S-100 was high. The nature of these proteins has not been determined. The peak next to S-100 protein is probably the 14-3-2 protein earlier identified as protein 4 and 5, and described by Hydén and Lange (1970) as being specifically related to learning.

The radioactivity values were obtained after ultrasonication of the incubated homogenized tissue. Ultrasonication is known to release newly synthesized soluble proteins from the particulate fraction (von der Decken and Campbell 1964) and, thereby to increase the concentration of the soluble proteins.

To obtain a high incorporation of amino acids into protein the pH of the incubation system, and the amount of slices per volume were found to be of greatest importance. The transport of  $^3\text{H}$ -leucine into cells was not a limiting factor of the system. The uptake was rapid and levelled off with time of incubation. Uptake of amino acids by rat brain slices has been reached in detail (Jones and McIlwain 1971). At least 30 min were required at 37° to

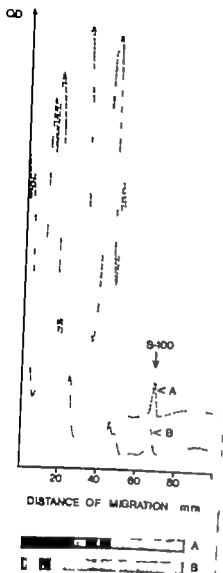


Fig. 7



Fig. 8

Fig. 7. Disc-electrophoretic patterns of soluble proteins from mouse brain. A) Soluble fraction obtained at 60% saturation of  $(\text{NH}_4)_2\text{SO}_4$ . B) Soluble fraction obtained at 100% saturation of  $(\text{NH}_4)_2\text{SO}_4$ . The amount of protein added to the electrophoresis tubes corresponded to 300 mg wet weight of tissue. The proteins were stained with Amadoblack 10 B, destained and the pattern measured in a microdensitometer.

Fig. 8. Immunodiffusion of S-100 protein. The middle well contained antibodies to beef S-100 protein, the outer wells the antigen recovered electrophoretically from polyacrylamide gels. The protein concentration of the antigen was 0.35 mg/ml.

### Discussion

In the present studies the synthesis of soluble acidic proteins *in vitro* by slices from brain of rats and mice is clearly demonstrated. The proteins were separated by polyacrylamide gel electrophoresis. The most acidic band migrating in front was shown to contain S-100 protein. There is a possibility of a glycoprotein being present in the same band (Haglid and Stavros, 1973) although treatment of the gels with 50% methanol did not seem to remove any protein. The type of the 1-dimensional electrophoresis used here with 2 spacer gels is to be compared

- Levy, E. The biochemistry of learning: An evaluation of the role of RNA and protein. *Ann. Rev. Biochem.* 1969 **38**, 606-644.
- Levy, K. G. and D. STAYROU, Water-soluble and pentanol-extractable proteins in human brain normal tissue and human brain tumours, with special reference to S-100 proteins. *J. Neurochem.* 1973 **20**, 1523-1532.
- Levy, K. G., L. RONALDSE and D. STAYROU, Purification of soluble glycoproteins from human nervous tissue and liver. *J. Neurochem.* 1973 **24**, 1053-1057.
- Levy, J. H. and R. E. WALLACE, Relation of oxygen and temperature in the preservation of tissue by nitrogen. *Proc. Soc. exp. Biol. (N.Y.)* 1949 **71**, 196-200.
- Levyman, H. R. Synthesis and degradation of brain-specific proteins (S-100 proteins) by clonal cultured brain glial cells. *J. Biol. Chem.* 1971 **246**, 7569-7571.
- Levy, T. and A. SCHWARTZ, Analogies in protein pattern and conformation among biosomes from different classes of vertebrates. *Comp. Biochem. Physiol.* 1971 **40 B**, 1011-1027.
- Levy, K. and P. W. LANGE, Correlation of the S-100 brain protein with behaviour. *Exp. Cell Res.* 1970 **62**, 125-132.
- Levy, D. A. and H. McILWAIN, Amino acid distribution and incorporation into proteins in isolated, electrically-stimulated cerebral tissues. *J. Neurochem.* 1971 **18**, 41-58.
- Levyman, E. and H. G. WITTMANN, Ribosomal proteins: VI Preparation of polycrylamide gels for electrophoresis as applied to the isolation of ribosomal proteins. *Anal. Biochem.* 1969 **30**, 132-141.
- Lowry, O. H., N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 1951 **193**, 265-275.
- Low, R. J. and G. D. NOVILL, Measurement of the incorporation of radioactive amino acids into proteins by filter paper disc method. *Arch. Biochem.* 1961 **94**, 48-52.
- Lyden, R. S. and H. HYDEN, A study of specific brain proteins on the semi-micro scale. *J. Neurochem.* 1966 **11**, 423-433.
- McILWAIN, H. and H. L. BUDDE, Techniques in tissue metabolism. I. A mechanical chopper. *Biochem. J.* 1953 **51**, 413-420.
- Moore, R. W. A soluble protein characteristic of the nervous system. *Biochem. Biophys. Res. Commun.* 1965 **19**, 238-244.
- Wass, B. W. and D. MCGAROCK, Chromatographic and electrophoretic fractionation of soluble proteins of brain and liver. *J. Biol. Chem.* 1965 **240**, 1647-1654.
- NATHANSON, D. In *Handbook of Experimental Immunology*, Weiss, D. M. (ed.) Blackwell Scientific Publications Oxford, 1967, 655-706.
- NEIL, J. In *Cell and Tissue Culture*, E. & S. Livingston, Edinburgh and London, 1970, 104.
- RONALDSE, L., K. G. LEVY, B. KARLSON and L. RONALDSE, Purification and characterization of soluble glycoproteins from rat brain (GM 30-C). *FEBS Letters* 1974 **38**, 143-146.
- ROBERTS, K., In *Densitische-Chromatographie*, Verlag Chemie GmbH, Weinheim/Bergstr. 1962, 32-36.
- ROBERTS, J. C. and F. C. KATZ, Microtechnique for determining the specific activity of radioactive intracellular lipids and applications to in vivo studies of protein synthesis. *J. Biol. Chem.* 1971 **246**, 6480-6482.
- STAYROU, D. and A. LUTKE, The distribution of amino acids, Na<sup>+</sup> and K<sup>+</sup> from surface to centre in coronal slices of mouse brain. *J. Neurochem.* 1974 **22**, 977-985.
- STAYROU, D., G. VINCENY, G. GORDON and P. MANDAL, Purification and some properties of S-100 protein fractions from sheep and pig brains. *J. Neurochem.* 1971 **18**, 429-432.
- STAYROU, D. A. and A. VON DER DECKEN, Incorporation of radioactive amino acids into proteins of brain slices from rat and mouse. *17th Intern. Congr. Biochem. Stockholm Abstracts* 1973, 154.
- STAYROU, D. M. and J. B. MILLER, Activation of the amino acid-incorporating system of rat liver slices upon incubation. *J. Biol. Chem.* 1955 **217**, 569-577.
- STAYROU, D. M., C. C. YOUNG and B. W. MOORE, Synthesis of brain-specific protein (S-100 protein) biosynthetic cell-free system programmed with cerebral polyosomal messenger RNA. *Proc. nat. Acad. Sci. (Wash.)* 1972 **69**, 2324-2330.
- STAYROU, D. M., H. HERSHMAN and L. LEVY, Appearance of brain specific antigen (The S-100 protein) during human fetal development. *J. Neurochem.* 1970 **17**, 247-251.

incorporation of radioactive amino acids into protein continues after that equilibrium between medium and slices is reached (Dunlop, van Elden and Lajtha 1974). Under the conditions used in the present investigation protein synthesis continued for another 150 min.

It has been reported (Folbergrová 1966) that an amino acid mixture added to a system decreased rather than increased the incorporation of radioactive amino acids into protein. In the present results an inhibition was observed at higher concentrations of radioactive amino acids. Metabolic conversion of radioactive amino acids being high even in brain (Banker and Cotman 1971) was shown to be low in cerebral slices after 120 min of incubation (Sershen and Lajtha 1974). A similar low conversion was obtained here with  $^{14}\text{C}$  leucine was analyzed after 5 hours of incubation.

The present method permits processing of amounts of brain tissue adequate for investigation of the synthesis of specific acidic proteins. It can be applied to studies of changes in the synthesis of these proteins caused by alterations in the physiological condition of the animal. Changes in the environment of the animal have been shown to affect protein- and RNA metabolism in the brain (von der Decken and Wronski 1971, von der Decken and Andersson 1972). Further it is known that both these processes are involved in brain functions such as learning and memory (for ref., see Glassman 1969).

The work was supported by grants from the Swedish Medical Research Council (project No. 4.66) and the Swedish Nutrition Foundation.

The authors are much indebted to Dr K. G. Haglid for discussion and his interest and the gift of purified S-100 protein and specific antibodies to beef S-100 protein.

The excellent technical assistance of Mrs C. Olgar is gratefully acknowledged.

## References

- BANKER, G. and C. W. COTMAN, Characteristics of different amino acids as protein precursors in mammalian brain: advantages of certain carboxyl-labelled amino acids. *Arch. Biochem.* 1971, 142, 565-573.
- BRAY, H. G. A simple efficient liquid scintillation counter for counting aqueous solutions in a liquid scintillation counter. *Analyt. Biochem.* 1960, 1, 279-285.
- CAMPBELL, P. N. and N. E. STONE, The synthesis of serum albumin and tissue proteins in slices of rat liver and liver tumor. *Biochem. J.* 1957, 66, 19-31.
- CHICKO, T. J. and B. W. MOORE, Transfer of the brain specific protein S-100. *Science* 1970, 169, 1333-1335.
- COHEN, P. P. In *Membrane Technique and Tissue Metabolism* (Umbreit, W. W., B. R. H. and S. S. J. F. (eds). Burgess Publishing Co., Minneapolis 15, Minn., U.S.A. 1951) 119.
- DAVIS, B. J. Disc-electrophoresis II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* 1964, 121, 404-427.
- VON DER DECKEN, A. Modification of the *in vitro* amino acid incorporation capacity of rat liver after *in vivo* and *in vitro* treatments. *Eur. J. Biochem.* 1968, 4, 87-94.
- VON DER DECKEN, A. and P. N. CAMPBELL, The effect of ultrasonic vibrations on the protein-synthesis activity of microsome preparations from rat liver. *Biochem. J.* 1964, 91, 195-201.
- VON DER DECKEN, A. and A. WRONSKI, Protein synthesis *in vitro* in rat brain after short-term protein starvation and refeeding. *J. Neurochem.* 1971, 18, 2383-2388.
- VON DER DECKEN, A. and G. M. ANDERSSON, Effect of protein intake on DNA-dependent RNA polymerase activity and protein synthesis *in vitro* in rat liver and brain. *Ann. Rep. Intern.* 1972, 5, 413-419.
- DEBROUWER, E., Ultrastructure and cytochemistry of the synaptic region. *Science* 1967, 156, 907-914.
- DUNLOP, D. S., W. VAN ELDEN and A. LAJTHA, Measurements of rates of protein synthesis in rat brain slices. *J. Neurochem.* 1974, 22, 821-830.
- FOLBERGROVÁ, J. Incorporation of labelled amino acids into protein of brain cortex slices *in vitro* in the presence of other non-radioactive amino acids. *J. Neurochem.* 1966, 13, 553-564.

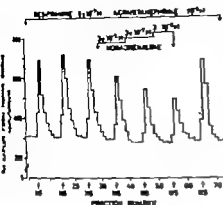


Fig. 1.

Fig. 1. Superfused human oviduct, preloaded with  $^3\text{H}$ -NA. Effect of NA on the outflow of tracer in response to transverse stimulation (NS), 150 pulses at 5 Hz. Time in min—fraction numbers.

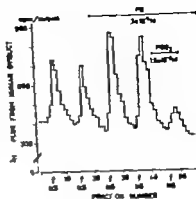


Fig. 2.

Fig. 2. Superfused human oviduct, preloaded with  $^3\text{H}$ -NA. Effects of phenylephrine (PBA) and prostaglandin  $\text{E}_2$  (PGE $_2$ ) on the outflow of tracer in response to transverse stimulation (NS), 150 pulses at 5 Hz. Time in min—fraction numbers.

transmitter release in the presence of uptake blockers suggest activities on presynaptic receptors.

Isoprenaline ( $4.7 \cdot 10^{-6}$ – $2.4 \cdot 10^{-5}$  M) produced inconsistent effects on evoked release of  $^3\text{H}$ -NA, when the above experimental design was used (presence of uptake blockers and stimulation at 5 Hz). Since the lack of effect might be due to the presumed presynaptic  $\beta$ -receptors already being saturated, further experiments were therefore performed according to the schedule introduced by Langer (1975), namely low frequency stimulation (1 Hz, 60 pulses), no uptake blockade, and low doses of isoprenaline ( $4.7$ – $9.5 \cdot 10^{-6}$  M). Using this protocol, isoprenaline consistently enhanced the evoked release of  $^3\text{H}$ -NA (Fig. 3). The elicited increase by isoprenaline ( $0.5 \cdot 10^{-6}$  M) of  $^3\text{H}$ -NA release resulting from transverse stimulation was  $48.0 \pm 15.1\%$  (mean  $\pm$  S.E., 6 expts,  $p < 0.05$ ). The enhancing effect of this dose of isoprenaline was reduced to  $6.3$ – $4.2\%$  in the presence of propranolol ( $10^{-6}$  M). In most cases propranolol itself had no effect on evoked release of  $^3\text{H}$ -NA, although in some cases it caused a slight to moderate inhibition. Enhancement of evoked transmitter release by isoprenaline, the effect of which is annulled by propranolol, suggests activation of presynaptic  $\beta$ -receptors operating at low catecholamine levels.

During recent years data have accumulated indicating that NA release from adrenergic nerve terminals, in many tissues may be subject to feed back control by PGE (Hedqvist 1976) and by presynaptic  $\alpha$ - and  $\beta$ -adrenoceptors (Starke 1972, Enero *et al.* 1972, Langer 1975, Späth and Brundin 1975). The present study and a previous report (Moewad *et al.* 1975) provide circumstantial evidence to show that NA release from the adrenergic nerves of the human oviduct may be controlled by any of these three mechanisms. If present *in vivo* two of them should be considered depressant and operate through activation of PGE and presynaptic  $\alpha$ -adrenoceptors respectively and the third to mediate positive feed back via activation of presynaptic  $\beta$ -receptors. It is in addition conceivable that the presynaptic

## Presynaptic $\alpha$ - and $\beta$ -adrenoceptor Mediated Control of Noradrenaline Release in Human Oviduct

By

P. HEDQVIST and A. MOAWAD

In the human oviduct, which is richly innervated by adrenergic nerve fibres, particularly in the isthmus, muscular effects of secreted noradrenaline (NA) are mediated by excitatory  $\alpha$ - and inhibitory  $\beta$ -adrenoceptors (Owman *et al* 1967, Rosenblum and Stein 1966). The relative activities of these receptors appear to be under hormonal control since oestrogen predominance enhances  $\alpha$ -adrenoceptor activity while progesterone elevates that of  $\beta$ -adrenoceptors (Moawad and Kim 1974).

In the search for presynaptic mechanisms controlling adrenergic transmission in the human oviduct, it has recently been shown that NA release by nerve activity is effectively suppressed by prostaglandin  $E_2$  ( $PGE_2$ ) (Moawad *et al* 1975). In the present study we wish to report data indicating that  $\alpha$ - and  $\beta$ -adrenoceptor agonists may negatively respectively positively affect the release of NA in this organ.

The isthmus part of human oviducts, 1½–2 cm length, was preincubated for 1 h in Tyrode containing 2.5  $\mu$ Ci/ml H-(+)-NA (spec. act. 3.8 Ci/mmol, M.E.N.). It was then mounted in a 2 ml bath and superfused with Tyrode at a rate of 1.5 ml/min. The composition of the Tyrode was (conc. in mM): Na 136.7, KCl 2.7,  $CaCl_2$  1.3,  $MgCl_2$  1.0,  $NaHCO_3$  11.9,  $NaH_2PO_4$  0.4, glucose 5.5, ascorbic acid 0.1 and was kept at 37°C and was gassed with 5%  $CO_2$  in  $O_2$ . The organ was transmurally stimulated by sector platinum electrodes in the wall of the bath and Grass S4 stimulator delivering trains of biphasic pulses (5 Hz, 1 ms, supramaximal voltage) at 10–15 min intervals. The superfusate was divided into one sample and the radioactivity was determined by counting 0.5 ml aliquots in a Packard scintillation spectrometer using 10 ml Instagel as counting medium. Quenching was monitored by internal standard. It has previously been shown that the bulk of tracer released by transmural stimulation consists of H-NA (Moawad *et al* 1975).

In the presence of desipramine ( $5 \cdot 10^{-6}$  M) and normetanephrine ( $10^{-6}$  M), administration in order to prevent uptake and uptake<sub>2</sub>, NA dose-dependently and reversibly inhibited the release of H-NA evoked by transmural stimulation (5 Hz, 150 pulses) (Fig. 1). The percentage inhibition of H-NA release by NA  $3 \cdot 10^{-6}$  M and  $3 \cdot 10^{-5}$  M was  $23.0 \pm 7.8$  and  $39.3 \pm 7.9$  respectively (means  $\pm$  S.E., 4 expts,  $p$ -values  $< 0.05$ ). On the other hand phenox benzamine (PBA),  $3 \cdot 10^{-6}$  M approximately caused a 3-fold increase of evoked H-NA release (Fig. 2). Notably the inhibition by  $PGE_2$  of evoked H-NA release was markedly reinforced in the presence of PBA. Inhibition by NA and enhancement by PBA of evoked



COMMUNICATIONS

C 1

**Prostaglandins and Post-Ischemic Muscular Vasodilation**

By Å. WEDQVIST and Å. KILBOM. *Department of Clinical Physiology Karolinska Institute Sörfjärder Hospital, 11283 Stockholm Sweden*

Prostaglandins of the E series (PGE) are powerful vasodilators. Recently it has been shown that hypoxia is a potent stimulus of PGE synthesis in a mammalian heart muscle (1) and that PGEs are possible mediators of reactive hyperemia (2). We have tested the hypothesis that endogenous PGEs take part in the vasodilation, and thus hyperemia, following arterial occlusion of the human forearm. Healthy volunteers, five men and five women, took part in the study. Forearm blood flow was measured using venous occlusion strain-gauges. Muscle blood samples for PGE analysis were obtained through a catheter inserted in a medial cubital vein. The content of PGE in plasma samples was absorbed on Amberlite XAD-2 and further purified using conventional lipid extraction procedures. Quantitative and qualitative analysis of the purified plasma extracts was performed by using bioassay and thin layer chromatography.

Each subject was studied twice, with 1 h interval. Initially the subject rested comfortably in a supine position, and the arterial blood flow was measured repeatedly after five minutes. Following this, arterial occlusion was applied with a pneumatic cuff on the upper arm. After five minutes, occlusion was released and the blood flow measured repeatedly during the following 24 min. Blood samples for PGE analysis were collected at rest and during the first 2 min after ischemia. Subsequently 100 mg of indomethacin (Indometacil, Sharp and Dohme) was administered rectally and after one hour the procedures of blood flow measurements and blood sampling were repeated.

The mean resting arterial blood flow was  $3.7 \pm 0.5$  (mean  $\pm$  S.E.  $n=10$ ) ml/100 g tissue min. No sex difference was obtained nor was the resting blood flow influenced by indomethacin. Following 5 min of arterial occlusion, marked increase in the blood flow was observed, both prior to and following administration of indomethacin. The concentration of PGE increased after ischemia. However the duration and the magnitude of the hyperemia was significantly decreased in the presence of indomethacin. In parallel the PGE concentration after ischemia was lower after indomethacin treatment.

Since administration of indomethacin in parallel decreased the magnitude of the reactive hyperemia and the concomitant release of PGE into the vein from the ischemic tissue we conclude that endogenous PGE, formed during tissue ischemia, take part in the development of muscular reactive hyperemia.

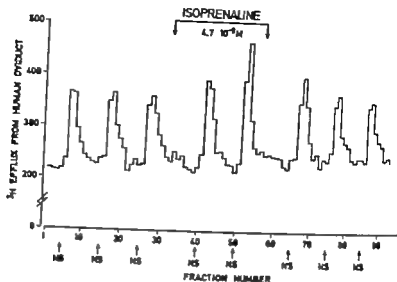


Fig. 3 Superfused human oviduct, preloaded with  $^3\text{H}$  NA. Effect of isoprenaline on the release of  $^3\text{H}$  NA in response to transmural stimulation (NS), 60 pulses at 1 Hz. Time in min = fraction numbers.

activities of PGE and adrenoceptors are themselves subject to variation, due to the hormonal conditions, as are their activities at the effector cell level (Moawad and Kim 1974, Moawad *et al.* 1975).

Supported by grants from the Swedish Medical Research Council, project No 04X-4342.

## References

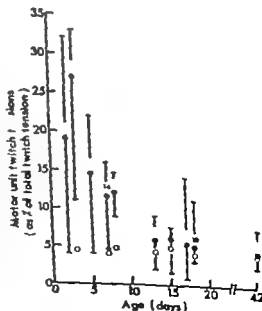
- ENERO, M. A., S. Z. LANGER, R. P. ROTHLEN and F. J. E. STEFANO, Role of the  $\alpha$ -adrenoceptor in regulating noradrenaline overflow by nerve stimulation *Br J Pharmacol* 1974, 44 672-682.
- HEDQVIST P. Prostaglandin action on transmitter release at adrenergic neuroeffector junctions. In *Advances in Prostaglandin and Thromboxane Research*, Vol. 2, eds B. Samuelsson and R. Paoletti, pp. 357-366. Raven Press, New York 1976.
- LANGER, S. Z., Prejunctional regulatory mechanisms for noradrenaline release elicited by nerve stimulation. In *Chemical Tools in Catecholamine Research* ed T. Malmfors, North-Holland Publishing Co., Amsterdam 1975 (in press).
- MOAWAD, A. H. and M. KIM, Plasma sex steroid levels and the in vitro response of the uterine portion of human oviduct to catecholamines. *Gynec Int* 1974, 5 19.
- MOAWAD A., P. HEDQVIST and M. BYGDEMANN, Noradrenaline release following nerve stimulation and its modification by prostaglandin  $E_2$  in human and rabbit oviduct. *Acta ph. med. scand.* 1975, 93 142-144.
- OWMAN Ch. E. ROSENKRANTZ and N. O. SJOBERG, Adrenergic innervation of the human female reproductive organs: a histochemical and chemical investigation. *Obster Gynecol* 1967, 30, 763-773.
- ROSENBLUM, I. and A. A. STEIN, Autonomic responses of the circular muscles of the isolated human Fallopian tube. *Amer J Physiol* 1966, 210, 1127-1129.
- STARKER, K., Influence of extracellular noradrenaline on the stimulation-evoked secretion of noradrenaline from sympathetic nerves: evidence for an  $\alpha$ -receptor-mediated feed-back inhibition of noradrenaline release. *Neuro-Schmidberg's Arch. Pharmacol.* 1972, 275 11-13.
- STJÄRNE, L. and J. BRUNDIN, Dual adrenoceptor-mediated control of noradrenaline secretion from human vasoconstrictor nerves: facilitation by  $\beta$ -receptors and inhibition by  $\alpha$ -receptors. *Acta ph. med. scand.* 1975, 94 139-141.

# Large-scale Reduction in Motoneurone Peripheral Fields during Post-natal Development in Rat

By M. C. BROWN, J. H. S. JANSEN and P. VAN ERSSEN. *Institute of Physiology University of Oslo, Norway*

The end-plate potential (e.p.p.) in curarized newborn rat muscles shows 2 or more components that are elicited at different strengths of nerve stimulation (Redfern 1970). The following experiments on neonatal rat soleus muscles support the suggestion of Bennett and Pettigrew (1974) that a convergence of several axons to a single synaptic spot on each muscle fibre accounts for these multicomponent e.p.p.s. Only a single spot of histochemical stain was seen on individual stained and teased muscle fibres (confirming Jänigk and Zelená 1966), but all components of the e.p.p. were reversibly increased in amplitude and time course after application of prostigmine. There was no sign of electrical coupling between adjacent muscle fibres impaled with separate microelectrodes. Finally components of the e.p.p. were equally depressed by iontophoretically applied doses of 300 nM tubocurarine that desensitized receptors within about 50  $\mu$ m of the acetylcholine pipette. Multiple innervation disappeared during the second week after birth: after day 15 less than 5% of fibres had multiple inputs. We measured the number of motor units that could be isolated in ventral root filaments and the tension produced by single units. The number of motor units remained roughly constant at about 25, but (see Fig. 1) their mean twitch tension, expressed as percentage of the total twitch tension, declined dramatically between days 2-3 (mean 23%) and days 15-18 (mean 5.4%). A similar but smaller change

Fig. 1. Average twitch tensions of motor units (filled circles,  $\bullet$ ) expressed as percentage of total twitch tension and the standard error (vertical bars) found at various ages in the rat soleus muscle. Ventral root motor units containing between one and five cells were stimulated while recording impulses from the soleus nerve. Isometric twitch contractions from the soleus muscle and its nerve dissected back to the roots L5 and L6 and bathed in oxygenated 10 mM  $\text{Ca}^{++}$  Ringers solution fixed with Tris at room temperature. Open circles ( $\circ$ ) give the average of motor units that would be expected if the total number of units found if there were no multiple innervation. The number of motor units was estimated individually for each muscle.



1. WENNMALM, Å. PHAM-HUU-CHANH and M. JONSTAD *Nature (Lond)* (in press).
2. KENT K M et al. *Physiologist* 1973 16 361

## C 2

## Recordings of Single Unit Activity from Cutaneous Nerve Fibres in Man

By H. HÄMÄLÄINEN, T. JÄRVILEHTO and P. LAURINEN, Department of General Psychology, University of Helsinki, Finland

Recording of single unit activity from cutaneous nerves in man presents a new approach to the study of peripheral neural mechanisms underlying cutaneous sensations (Knit and Vallbo 1970). In the present work characteristics of single units in the human nerve were studied with the purpose of determining the neural input necessary for touch sensation.

Tungsten microelectrodes (tip diameter 1-3  $\mu\text{m}$ ) were inserted into the radial nerve at the wrist by means of a micromanipulator. Single unit potentials were amplified on magnetic tape and analyzed in a computer.

Preliminary results are based on recordings from 70 single units. Four units did not respond to the manipulation of the skin. One of them was affected by movements of the index finger and 3 units by cigarette smoking or deep breathing. The former unit had a regular spontaneous discharge (mean of interval histogram  $\bar{X}_1=137$  ms, standard deviation,  $s=34$  ms). Pressing of the finger downwards caused ceasing of the discharge which reappeared with lifting of the finger. One unit fired in very regular bursts of 10-40 impulses separated by pauses of 1-4 s (within burst  $\bar{X}_1=50$  ms,  $s=20$  ms). Smoking of a cigarette resulted in longer intervals both within and between the bursts and in marked reduction of the number of impulses. Deep breathing reduced firing frequency of 2 units, one of them firing spontaneously in bursts (within burst  $\bar{X}_1=19$  ms,  $s=9$  ms, inter-burst intervals, 0.1-0.5 s), the other having a regular discharge ( $\bar{X}_1=56$  ms,  $s=5$  ms). The latter units resemble sympathetic units described by Dellus et al. (1972).

Sixteen units responded to mechanical stimulation of the skin. According to the response to sustained pressure, 5 units could be classified as rapidly adapting and 11 as slowly adapting (SA) ones. Conduction velocities of 4 SA-units measured by applying electrical pulses to receptive fields ranged from 12-32 m/s. The thresholds of the SA-units for a few impulses to appear ranged from 0.1-1.2 g, which corresponded well to the report of Knit (1970) on threshold sensation. An average discharge of 10-20 imp/s, however, caused usually only weak touch sensation and for a moderate sensation impulse frequencies up to 40 imp/s were necessary. The results suggest that a few impulses in a mechanoreceptive fibre may suffice for detection of a tactile stimulus, but a relatively large number of impulses is necessary for a distinct touch sensation.

## References

- DELLUS, W., K. B. HAGBARTH, A. HONGELL and B. G. WALLIN, *Acta physiol. scand.* 1972, 84, 65-81.  
KNIT, M. and A. B. VALLBO, *Acta physiol. scand.* 1970, 80, 178-195.

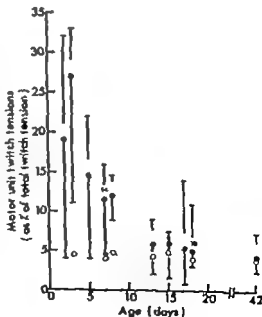
# Large-scale Reduction in Motorneurone Peripheral Fields during Post-natal Development in Rat

By M. C. BROWN, J. K. S. JANSSEN and D. VAN ERSSEN. *Institute of Physiology University of Oslo, Norway*

The end-plate potential (e.p.p.) in curarized newborn rat muscles shows 2 or more components that are elicited at different strengths of nerve stimulation (Redfern 1970). The following experiments on neonatal rat soleus muscles support the suggestion of Bennett and Pettigrew (1974) that a convergence of several axons to a single synaptic spot on each muscle fibre accounts for these multicomponent e.p.p.s. Only a single spot of cholinesterase was seen on individual stained and teased muscle fibres (confirming Libonka and Zelinka 1966), but all components of the e.p.p. were reversibly increased in amplitude and time course after application of prostigmine. There was no sign of electrical coupling between adjacent muscle fibres impaled with separate microelectrodes. Finally all components of the e.p.p. were equally depressed by iontophoretically applied doses of micycloheximide that desensitized receptors within about 50  $\mu$ m of the acetylcholine pipette.

Multiple innervation disappeared during the second week after birth: after day 15 less than 5% of fibres had multiple inputs. We measured the number of motor units that could be activated in ventral root filaments and the tension produced by single units. The number of motor units remained roughly constant at about 25, but (see Fig. 1) their mean twitch tension, expressed as a percentage of the total twitch tension, declined dramatically between days 2-3 (mean 23%) and days 15-18 (mean 4.4%). A similar but smaller change

Fig. 1 Average twitch tensions of motor units (filled circles,  $\bullet$ ) expressed as a percentage of total twitch tension and the range in units (vertical bars) found at various ages in rats (vertical bars) found at various ages in the rat soleus muscle. Ventral root filaments containing between one and five motor units were stimulated while recording impulses from the soleus nerve and recording twitch contractions from the muscle in an isolated preparation containing the muscle and its nerve dissected back to spinal roots L5 and L6 and bathed in oxygenated 10 mM  $\text{Ca}^{++}$  Ringers solution buffered with Tris at room temperature. Open circles ( $\circ$ ) give the average size of motor units that would be expected from the total number of units found if there were no multiple innervation. The number of units whose tension was examined individually is given for each muscle.



## References

1. WENNEMÄLK, Å., PHAM-HUU-CHANH and M. JUNSTAD, *Nature (Lond)* (in press).
2. KENT, K. M. et al *Physiologist* 1973 16 361

## C 2

### Recordings of Single Unit Activity from Cutaneous Nerve Fibres in Man

By H. HAMÄLÄINEN, T. JÄRVILEHTO and P. LAURINEN. *Department of General Psychology, University of Helsinki, Finland*

Recording of single unit activity from cutaneous nerves in man presents a new approach to the study of peripheral neural mechanisms underlying cutaneous sensations (Käster and Vållbo 1970). In the present work characteristics of single units in the human nerve were studied with the purpose of determining the neural input necessary for touch sensation.

Tungsten microelectrodes (tip diameter 1-3  $\mu\text{m}$ ) were inserted into the radial nerve at the wrist by means of a micromanipulator. Single unit potentials were amplified, stored on magnetic tape and analyzed in a computer.

Preliminary results are based on recordings from 70 single units. Four units did not respond to the manipulation of the skin. One of them was affected by movements of the index finger and 3 units by cigarette smoking or deep breathing. The former unit has a regular spontaneous discharge (mean of interval histogram  $\bar{X}_1 = 137$  ms, standard deviation  $s = 34$  ms). Pressing of the finger downwards caused ceasing of the discharge which reappeared with lifting of the finger. One unit fired in very regular bursts of 10-40 impulses separated by pauses of 1-4 s (within burst  $\bar{X}_1 = 50$  ms,  $s = 20$  ms). Smoking of a cigarette resulted in longer intervals both within and between the bursts and in marked reduction of the number of impulses. Deep breathing reduced firing frequency of 2 units, one of them firing spontaneously in bursts (within burst  $\bar{X}_1 = 19$  ms,  $s = 9$  ms, inter-burst-intervals, 0.5 s), the other having a regular discharge ( $\bar{X}_1 = 56$  ms,  $s = 5$  ms). The latter units resemble sympathetic units described by Delfus et al (1972).

Sixteen units responded to mechanical stimulation of the skin. According to the response to sustained pressure, 5 units could be classified as rapidly adapting and 11 as slowly adapting (SA) ones. Conduction velocities of 4 SA-units measured by applying electrical pulses to receptive fields ranged from 12-32 m/s. The thresholds of the SA-units for a first impulse to appear ranged from 0.1-1.2 g, which corresponded well to the report of threshold sensation. An average discharge of 10-20 imp/s, however, caused usually only weak touch sensation and for a moderate sensation impulse frequencies up to 40 imp/s were necessary. The results suggest that a few impulses in a mechanoreceptive fibre are sufficient for detection of a tactile stimulus, but a relatively large number of impulses is necessary for a distinct touch sensation.

## References

- DELFIUS, W., K.-E. HAGBARTH, A. HOGRELL and B. G. WALLIN, *Acta physiol. scand.* 1972. 84 65-81  
KÄSTER, M. and A. B. VÅLLBO, *Acta physiol. scand.* 1970. 80 178-195

was totally dependent on the presence of  $Mg^{2+}$  in the medium. There was no effect of the % of vasopressin in the filtrate either in a medium containing 1 mM EGTA or 1 mM  $Ca^{2+}$ .

In these experiments it would appear that the ATP-induced emptying of secretory vesicles is, at least in part, an enzyme catalysed reaction.

### References

1. and D. B. Horvath, *Biochem. J.* 104 1967 1082-1083.  
2. M. and W. W. DOUGLAS, *Molecular Pharmacol.* 1968, 4 531-540.

### C 5

## 1 Control of Glycolysis and Oocyte Maturation in the Preovulatory Rat Ovarian

HILLENSDÖ, Department of Physiology University of Göteborg Sweden

Luteinizing hormone (LH) is known to induce several biochemical and morphological transformations in preovulatory follicles. Thus the hormone, by its steroidogenic pattern, causes resumption of oocyte meiosis ("oocyte maturation") and induces changes in the follicular wall eventually leading to its rupture. The mechanisms behind these effects are largely unknown. Concerning the induction of ovulation, several triggering factors within the follicle have been suggested. In the luteal and cyclic AMP certain energy substrates such as pyruvate-lactate are mediators of this LH-effect (Lindner *et al.* 1974).

Our studies have shown that gonadotropins stimulate cyclic AMP formation and release in isolated preovulatory rat follicles (Lindner *et al.* 1974 Nilsson 1974 Nilsson 1974). The aim of the present study was to examine oocyte maturation and glycolysis in preovulatory rat follicles exposed to gonadotropins *in vivo* or *in vitro*. Female rats were injected with 10 IU PMS when 30 days old leading to follicle maturation and subsequent endogenous release of LH in the afternoon of day 32. Time of LH surge, oocyte maturation and ovulation has been published (Hillensjö *et al.* 1974). Rats were killed before and after the LH surge on day 32, the ovaries removed and follicles isolated by microdissection. The follicles were incubated in Krebs bicarbonate buffer containing 5.5 mM glucose and 1% bovine serum albumin without hormone. The follicles were immersed in 0.5 ml medium in flasks gassed with 5%  $CO_2$ . After incubation the lactate concentration in the medium was analysed enzymically. The oocytes were recovered for direct inspection using Nomarski interference contrast microscopy. The occurrence of germinal vesicle breakdown (GVB) and morphological signs of oocyte maturation was recorded.

The first group of rats were sacrificed between 9.00-13.00, the follicles isolated and incubated. In control medium a linear lactate production was found during 0.5-6 h incubation. The oocytes did not mature. In presence of LH (NIH S18) 0.1-10  $\mu$ g/ml or FSH (NIH S10) 1-10  $\mu$ g/ml a pronounced stimulation of lactate production was seen. The oocytes also induced GVB in the oocytes. After 2 h of incubation a small proportion of oocytes showed GVB whereas 90-100% showed GVB after 4 h incubation.

has been reported in kittens (Bagust *et al* 1972). Our results suggest that the average of about 5 functional synapses on each muscle fibre shortly after birth and disappearance of multiple innervation occurs by means of a decline in the relative motor units with little change in their number.

### References

- BAGUST J. D. M. LEWIS, J. C. LUCK and R. A. WESTERMANN, *J. Physiol.* 1972 224 35P  
 BENNETT M. R. and A. G. PRITTIGREW *J. Physiol.* 1974 241 515-545  
 LUBINSKA L. and J. ZELANA, *Nature* 1966 210 34-41  
 RILDFERN W. A. *J. Physiol.* 1970 209 701-709

### C 4

#### Adenosine Triphosphate-induced Emptying of Isolated Bovine Neurohypophyseal Secretory Granule Contents

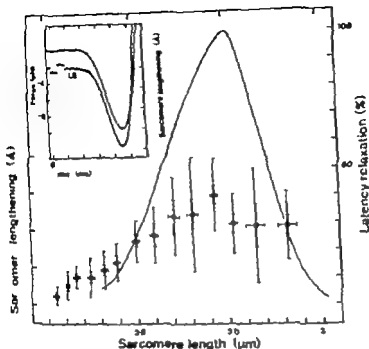
By J. T. RUSSELL and N. A. THORN *Institute of Medical Physiology C, University of Copenhagen, Denmark*

It has been reported that ATP can induce release of vasopressin from otherwise stable isolated neurosecretory granules (Polsner and Douglas 1968). However, it is not clear whether this is due to membrane instability caused by ATP or an enzymatic effect.

A crude secretory granule fraction was obtained from bovine neurohypophyses (Fraction III of Dean and Hope 1967). The granules were suspended in 2 ml of incubation medium (composition (mM): KCl 124.5, NaCl 5.0, CaCl<sub>2</sub> 0.001, MgCl<sub>2</sub> 3.0, Tris (hydroxymethyl) methyl 2-aminoethane sulphonic acid 70.0, pH 7.0) and aliquots were added to vessels with 70 or 2.5 ml of incubation medium (final protein concentration 200-300 µg/ml) and incubated at 37 or 5°C in a shaking water bath. The reaction was stopped by filtering 2.0 ml aliquots of the reaction mixture through millipore filters (pore size 0.22 µm). The filtrates were passed through Whatman DE81 ion exchange discs to remove ATP. The filtrate and an acetic acid (0.25%) extract of the filter were assayed for vasopressin activity. The results were expressed as % of total vasopressin in the 2 ml aliquot appearing in the filtrate.

In the absence of ATP approximately 10% of the total vasopressin appeared in the filtrate even after 60 min of incubation. When ATP was added to the medium, however, more than 50% of the hormone activity was found in the filtrate in 30 min. This effect of ATP was dose dependent, showing a threshold concentration of 0.2 mM and a maximal effect at 1.0 mM ATP. Inclusion of an ATP regeneration system further increased the % vasopressin in the filtrate to 80. This value was not different from the % hormone that could be released by vigorous sonication. Electron microscopic observation of the ATP-treated granules showed that they had totally lost their electron opaque cores but still possessed intact membranes. The percentage appearing in the filtrate was significantly lower at 5°C than at 37°C (79.8±2.8% at 37°C in 30 min, 51.9±4.1% at 5°C,  $p < 0.001$ ). N-ethylmaleimide, a known inhibitor of ATPase, blocked this ATP-induced emptying of granules at a conc. of 2 mM. The effect of ATP showed a pH optimum between 6.8-





the values of latency elongation shown with  $\pm 1$  S.D. (thin bar) and  $\pm 1$  S.E.M. (thick bar). Size of latency relaxation indicated by continuous curve. Insert: Latency relaxation and tension with stimulation waveforms added.

ing. The tension drop is a function of the slope stiffness of the series elasticity and of the sarcomere length changes.

supported by the Norwegian Research Council for Science and the Humanities.

#### References

- and O. STEV-K. LØRUM. *Acta physiol scand.* 1960 50 Suppl 175. 63-64  
 L.L. *J Physiol (Lond)* 1972 223 333-334

#### C 7

stration of Tissue Hyperosmolality in the Tip of Intestinal Villi during Sodium Ion Absorption

MATS JØDAL and OVE LUNDGREN. Department of Physiology University of Göteborg Sweden

we earlier proposed that a marked interstitial sodium concentration gradient along length of the cat intestinal villi was created during sodium absorption by a counter-current multiplication of sodium in the villi, resulting in a high tissue osmolality in the tips of villi (Haljamaa *et al.* 1973). This hyperosmolar compartment was proposed to be of importance also for the absorption of water particularly in the absence of or against a lumen-to-blood osmotic gradient.

The second group of rats were sacrificed between 18 00-19 00 OVB had then occurred *in vivo*. Lactate production of these follicles incubated in control medium were much higher (3-fold) than of corresponding follicles of the morning group yet addition of (0.1-10  $\mu\text{g/ml}$ ) resulted in a slight but significant increase.

The results show that properly characterized PMS-treated prepubertal rats constitute a convenient experimental model for studies of oocyte maturation and related biochemical phenomena. The finding that exogenous as well as endogenous gonadotropins in addition to the meiosis inducing action markedly enhanced follicle glycolysis suggests that these actions might be related. Further studies are needed in order to establish a causal relationship between these two events.

### References

- HILLEBRAND, T. A. BARNEA, L. NILSSON, H. HERLITZ and K. ÅHRÉN, *Endocrinology* 1974, 95, 1762-1766.  
 LINDNER, H. R. A. TRAFURI, M. E. LILBERMAN, U. ZOR, Y. KOCH, S. BAUMINGER and A. BARN, *Recent Progress in Hormone Research*, Ed. R. O. Greep, A. P. N. Y. 1974, 30, 79-138.  
 NILSSON, L., *Acta endocr. (Kbh.)* 1974, 77, 340-358.  
 NILSSON, L., S. ROSENBERG and K. ÅHRÉN, *Acta endocr. (Kbh.)* 1974, 77, 559-574.

### C 6

#### Early Mechanical Response in Isolated Frog Skeletal Muscle Fibres

By P. HAUGEN and O. STEN KNUDSEN, *Department of Biophysics, University of Copenhagen, Denmark*

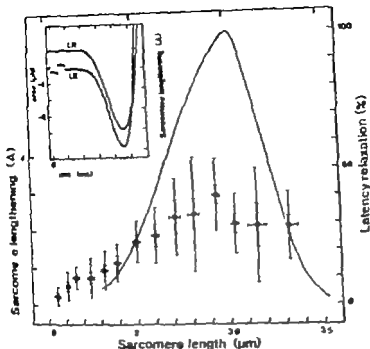
The latency relaxation and the latency elongation (the corresponding lengthening of sarcomeres) were investigated in single muscle fibres.

The latency elongation was measured by a laser diffraction technique using photoelectric difference detection to register small changes in the position of the first order spectrum resulting from the latency elongation. Sarcomere length changes as small as 0.1  $\mu\text{m}$  could be measured. The fibre was stimulated transversely to ensure synchronous excitation.

Latency relaxation was measurable at sarcomere lengths from 2.15  $\mu\text{m}$  to 3.5-3.7  $\mu\text{m}$  in contrast to Mulieri's (1972) findings: the amplitude of the tension drop passed through a sharp maximum which occurred at a sarcomere length of 3.0-3.1  $\mu\text{m}$ . Latency relaxation is dependent on overlap between thick and thin filaments. The effect of the sarcomere length on the time course agrees with the findings of Guld and Sten Knudsen (1960).

The latency elongation was not uniform along the fibre. In some regions maximum lengthening occurred either before or simultaneously with the maximum tension drop; in other regions the sarcomeres did lengthen further before contraction and might even be pulled further out by contraction of neighbouring sarcomeres. In the range from 2.3  $\mu\text{m}$  to 3.0  $\mu\text{m}$  the mean latency elongation increased by a factor of 3 while the latency relaxation increased 20 times.

In completely slack fibres where no drop in tension could be detected, latency elongation was observed down to 2.05  $\mu\text{m}$ . Thus, the latency relaxation is caused by an act



less values of latency elongation shown with  $\pm 1$  S.D. (thin bar) and  $\pm 1$  S.E.M. (thick bar) size of latency relaxation indicated by continuous curve. Insert: Latency relaxation and elongation with stimulation waveform added

arg. The tension drop is a function of the slope stiffness of the series elasticity and of the sarcomere length changes

is supported by the Norwegian Research Council for Science and the Humanities.

#### References

- C and O STEIN-KJØLLSEN, *Acta physiol scand* 1969, 50 Suppl. 175 63-64  
 H. L. J. *Physiol (Lond.)* 1972, 223 333-354

#### C 7

etration of Tissue Hyperosmolality in the Tips of Intestinal Villi during Sodium Ion Absorption

by MATS JODAL and OVE LUNDGREN, *Department of Physiology University of Göteborg Sweden*

an earlier proposed that a marked interstitial sodium concentration gradient along length of the cat intestinal villi was created during sodium absorption by a countercur multiplication of sodium in the villi resulting in a high tissue osmolality in the tips of villi (Haljand *et al* 1973). This hyperosmolar compartment was proposed to be of importance also for the absorption of water particularly in the absence of or against a lumen-to-blood osmotic gradient

According to the countercurrent hypothesis a small increase of plasma sodium concentration in the subepithelial villus capillaries caused by active sodium absorption, result a crossdiffusion of sodium from the subepithelial network into the central arterial vein and of water in the opposite direction. In the mentioned study this hypothesis substantiated by demonstrating that the sodium content per unit weight tissue protein 3-4 times higher at the villous tip than at the base

To obtain a more direct measure tissue hyperosmolality has now been determined by cryoscopy. During net water absorption from isotonic NaCl-glucose solution normo blood perfused intestinal segments were suddenly frozen in liquid nitrogen or isopentane. Intestinal slices 10  $\mu\text{m}$  thick were cut at  $-20^{\circ}\text{C}$  in a cryostat and the sections microscopically examined in a room at  $-10^{\circ}\text{C}$ . The temperature of the intestinal section was increased in a stepwise fashion and the temperature at which thawing occurred in different parts of the intestinal wall was determined. The villus tips were clearly the last intestinal structures to thaw and a gradient of freezing point depression was observed along the villi. The crypt sections thawed at the same temperature as the submucosa and muscularis corresponding to an osmolality of slightly above 300 mOsm/kg. In contrast freezing point depression in the villous tips corresponded to a tissue osmolality of about 1200 mOsm/kg. Thus the obtained results are in agreement with the proposed countercurrent multiplication hypothesis

### References

HALJAMAR, H. M. JODAL and O. LUNDÖREN *Acta physiol scand* 1973 **89** 580-593

### C 8

#### Osmolar Control of Extracellular and Plasma Volume in Hemorrhagic Hypotension

By JOHANNES JÄRHULT *Department of Physiology University of Lund Sweden*

The aim of the present investigation was to define blood osmolality changes during hemorrhagic hypotension, their underlying mechanisms and their importance for fluid volume redistribution between different body compartments in hypovolemia. The experiments were performed on anesthetized cats exposed to standardized rapid bleeding to a constant arterial pressure level of 50 mm Hg.

Hemorrhage caused a rapid and pronounced increase of arterial plasma osmolality which almost fully could be accounted for by a concomitant hyperglycemia. The hemorrhagic hyperglycemia was caused by glucose release from the liver, in turn mediated via different reflex mechanisms. By direct sympathetic nervous influence on the liver adrenergically released glucagon from the pancreas and by catecholamines emanating from the adrenal medulla. Quantitative investigations of capillary fluid exchange in skeletal muscle revealed a considerable transcapillary absorption of extravascular fluid into the blood stream during the first hour of hemorrhagic hypotension. Such fluid transfer occurred not only in the intact muscle region, but also after regional  $\alpha$ -adrenergic blockade and sympathectomy, showing that mechanisms other than the previously known adrenergic reflex resetting of the pre-/post-capillary resistance ratio contributed to the fluid a

tion. Critical analysis indicated that an osmotic mechanism, related to the arterial pressure (hyperglycemia), was responsible for the capillary fluid transfer in the excised muscle. Transcapillary absorption of extravascular fluid from skeletal muscle evoked by both these mechanisms is of great hemodynamic importance in hemorrhage as it helps to restore plasma volume. Extrapolation of the present observations to man suggests that a total extravascular fluid volume of about 600 ml would be reabsorbed into the blood stream from all skeletal muscle tissue within the first hour of orthostatic hypotension. The osmotic mechanism would be responsible for at least 50% of this effect.

It appears that the described osmolar fluid control is basically aimed at an overall reduction of extracellular fluid volume in hemorrhage causing a glucose-osmotic fluid shift withdrawal from the intracellular compartment. The consequent dilution of protein in the interstitial space of the tissues can help to explain why the osmotically and freely absorbed protein-poor extravascular fluid from skeletal muscle is retained for considerable length of time within the circulatory system. The efficiency of this mechanism depends on the interstitial protein concentration which is high before dilution. Recent studies have indicated that interstitial colloid-osmotic pressure indeed is significant and much higher than previously assumed (see Aukland 1973).

#### Reference

LELAND, K. Autoregulation of interstitial fluid volume. Edema-preventing mechanisms. *Scand. J. Clin. Lab. Invest.* 1973, 31: 247-254

#### C 9

**Normal Panting Reduces Oxygen Uptake in the Dik Dik, *Rhynchotragus dikdik***

By P. P. HOPPE, K. JOHANSEN, G. M. O. MALOY and V. MUSEWE. *Departments of Animal Physiology, University of Nairobi, Kenya, and Department of Zoophysiology, University of Aarhus, Denmark*

Study of oxygen uptake ( $\text{VO}_2$ ) during heat stress in the Dik Dik, a small ruminant species (2.5-7 kg) is of special interest because of its high metabolic rate and labile core temperature. The animal inhabits semi-arid bush country in East Africa and encounters intense radiative heat. Its practice of thermal panting has been reported earlier (Maloy 1973).

$\text{VO}_2$  was measured at thermoneutrality ( $T_a = 22-27^\circ\text{C}$ ) and during heat stress ( $T_a = 40-43^\circ\text{C}$ ) in a temperature controlled room. The animals were suspended in a harness and fitted with face masks during measurements. A Servomex  $\text{O}_2$  analyzer, gas flowmeters and pumps were used for the  $\text{VO}_2$  measurements. Respiratory rate, rectal and skin temperatures were continuously recorded. The results (Table 1) surprisingly reveal that  $\text{VO}_2$  during shallow rapid panting is generally less than during normal breathing at thermoneutrality. Measurements were only done if the animals accepted the face mask for 4-7 min periods and if the depth and rate of breathing continued unchanged.

Expt.	T	T	Breaths/min	VO <sub>2</sub> (ml O <sub>2</sub> STPD/g/h)	VO <sub>2</sub> % of value at thermoneutrality
1	21	39.0	18	0.55	100
	43	39.0	100	0.55	100
	43	39.2	210	0.58	105
2	23	38.3	70	0.57	100
	43	39.4	180	0.34	60
	43	39.8	120 230	0.46	81
	43	39.8	170 230	0.39	68
	28	38.2	30	0.50	88
3	21	38.0	4	0.54	100
	40	40.1	240	0.35	64
	40	40.3	168	0.34	63
	24	39.0	20	0.515	94
4	27	—	38	0.5	100
	37	40.4	42	0.68	130
	35	41.5	50	0.39	75
	35	41.7	330	0.30	58

A reduced VO<sub>2</sub> during panting has to our knowledge not hitherto been reported. It must be associated with a reduced alveolar ventilation in spite of the much elevated total ventilation. The efficiency of thermal panting in heat dissipation of the Dik Dik is unquestionable. A prolonged duration must, however, jeopardize the continued provision of O<sub>2</sub> for aerobic metabolism.

The behaviour pattern of the Dik Dik is suggestive that the described response may afford an emergency short term mechanism for intermittent heat dissipation which may enable this miniature antelope with a very low thermal inertia to survive brief periods of intensive heat stress.

#### References

G. M. O. MALORY *Proc. Roy. Soc. London B* 184: 167-178

#### C 10

**Tensile Strength of Gas Saturated Biological Liquids Exposed to Successive High Pressures**  
By LARS OLE KORNUM, LEIF BJØRNØ, PER KRAAG, CARL HELGE NIELSEN and POUL-ERIK PAULEV *Institute of Medical Physiology B, University of Copenhagen and Department of Fluid Mechanics, Technical University of Denmark*

A liquid under pressure is being saturated with a given gas—Argon, Nitrogen, Carbon dioxide and Air—by circulation through a column of gas exchangers. A sample of the gas saturated liquid is isolated in a test chamber, the volume of which may be increased by means of a moving piston. The piston motion is cyclical with a variable frequency. The pressure in the test chamber is measured by means of a capacitive pressure pick-up. When the volume (increase of the gas saturated liquid in the test chamber is being compensated by

to development of gas phase bubbles the pressure decrease will stop. The recording trace will show a pressure plateau or just a turn in the pressure-time course depending on the relationship between the velocity of the growth of the bubbles and depending on the pulse modes. The tensile strength revealed to be independent of the frequency of the cyclic pressure decrease within the frequency limits 1 Hz down to  $10^{-2}$  Hz. Most experiments were carried out at one single frequency of 0.5 Hz.

In the treatment of decompression sickness it has long been known that oxygen has a substantial beneficial effect in the last stages of recompression procedure. The biological mechanism implied is unknown (Elliott, Hallenbeck and Bove 1974 Royal Navy Diving Manual 1972, U.S. Navy Diving Manual 1973).

Measurements with the above method on different liquids saturated with different gases showed that biological liquids saturated by gases containing oxygen showed a remarkable higher resistance (approximately 3 times higher in pig serum) to bubble formation and a lower resistance to resolve by recompression.

Oxygen increases the surface activity of biological liquids. This effect may offer an explanation of the unknown biological mechanism stated above.

#### References

- ELLIOTT, D. H., J. M. HALLENBECK and A. A. BOVE, *Lancet* 1974 *ii* 1193-1199  
 Royal Navy Diving Manual, H. M. Stationery Office 1972.  
 U.S. Navy Diving Manual, Navships, Washington, D.C., 1973

#### C 11

##### Ion Transport in Non-acid Secreting Gastric Epithelium

By GUNMAR FLENETRÖM Department of Physiology and Medical Biophysics University of Uppsala, Sweden and Department of Physiology and Biophysics University of Alabama in Birmingham, USA

The gastric antral mucosa is of considerable physiological interest. This region separates the secretory epithelium of the gastric fundus from the absorptive epithelium of the small intestine and its properties are important in relation to control of gastrin release. The antral epithelium is composed mainly of surface epithelial cells while transport in the fundus is dominated by the secretion of hydrochloric acid, presumably originating from the parietal cells. The aim of the present study has been to examine ion transport in the antrum and the non-acid secreting fundus.

Well defined isolated *Necturus* antral mucosae were mounted in chambers. Flux studies showed that both sodium and chloride were actively transported sodium from the lumen to the nutrient (interstitial) side and chloride in the opposite direction. Only the sodium transport contributed to the electrogenic properties of the tissue as evidenced by abolition of the transepithelial electric potential difference on replacement of sodium with choline and the inhibitory effect of amiloride ( $10^{-6}$  M, luminal side) and ouabain ( $10^{-6}$  M, nutrient side) on the electrogenic properties and net sodium flux. Microelectrode studies showed a amiloride sensitive sodium conductance in the luminal cell membrane and potassium ar

chloride conductances in the nutrient cell membrane both cell membranes being permeable also to bicarbonate

Antral mucosae from *Necturus* and *Rana catesbeiana* were capable of alkalinizing the luminal side solution and this was arrested by inhibitors of tissue metabolism inhibited by acetazolamide. Addition of bicarbonate to the nutrient side increased the rate of alkalization and the transepithelial electric potential difference. An alkalization of the luminal solution with the same sensitivity to inhibitors as that in the antrum was found in fundic mucosae from *Rana catesbeiana* and *Rana temporaria* when acid secretion was either inhibited by thiocyanate or the  $H_2$  receptor antagonist Burimamide, or spontaneously absent. The rate of alkalization in both fundus and antrum amounted to approximately 10 percent of maximal fundic hydrogen ion secretion *in vitro*.

It is suggested that the alkalization may protect the luminal surface of the epithelium from intraluminal acid and that this might explain previously reported (Wenther *et al* 1965) ulcerogenic effects of acetazolamide.

### References

- FLENETRÖM G and G SACHS, *Amer J Physiol* 1975 (in press).  
 FLENETRÖM G In *Mechanisms of H<sup>+</sup> secretory processes* D. H. KASBEKAR, ed. Decker Inc. New York, 1975 (in press).  
 WENTHER, J. L., F. HOLLANDER and M. ALTAMIRANO, *Amer J Physiol*, 1965 209: 127-133.

### C 12

#### Aspects of the Central Interaction between Excitatory Chemoreceptor and Inhibitory Baroreceptor and Cardiac Receptor Influences

By GÖRAN WENNERGREN, ROD LITTLE and BENGT ÖBERG, *Department of Physiology, University of Göteborg, Sweden*

Our present knowledge of cardiovascular reflex control emanates mainly from experiments where individual reflexes have been separately studied while disturbing influences from other receptors have been eliminated. However, in most situations calling for cardiovascular adjustments, there is usually a simultaneous engagement of several receptors and the observed responses therefore constitute an integrated net effect of several influences (cf. Körner 1971). During hypoxia, for instance, there occurs a concomitant activation of arterial chemo- and baroreceptors and also of cardiac ventricular receptors (Thoren 1971). To analyse the vasomotor centre response to such an input profile of mixed excitatory (chemoreceptor) and inhibitory (baro- and ventricular receptors) influences, experiments were performed on anesthetized, artificially ventilated cats. Baro- and chemoreceptor reflexes were produced by perfusing the isolated carotid sinus regions at varying pressures with arterial or venous blood. Ventricular receptor afferents in the right cardiac nerve were electrically stimulated.

The results suggest that a simultaneous chemo- and baroreceptor activation implies a simple summation of excitatory and inhibitory influences on medullary vasomotor neurons, while the interaction between chemoreceptor and cardiac receptor influences



ner complex. Thus, the vasodilator responses to cardiac nerve stimulation were effectively suppressed by a concomitant chemoreceptor stimulation, provided that a primary reflex bradycardia occurred. In contrast, the bradycardia response to cardiac stimulation was essentially uninfluenced by alterations in chemoreceptor activity. Experiments with cats were exposed to severe hypoxia (ventilation with 3% O<sub>2</sub>, 4% CO<sub>2</sub>) leading to a marked reflex bradycardia and vasoconstriction. Interruption of the afferents from cardiac receptors eliminated the bradycardia response but did not suppress the vasoconstrictor response to hypoxia. This again demonstrates that while the chemoreceptors may contribute significantly to the reflex bradycardia during hypoxia, they are capable to exert their reflex vasodilator influences in the presence of a strong vagal stimulation. This interaction between the two reflexes may be particularly important during a situation in which a maintained strong vasoconstriction is urgently required despite a proposed activation of ventricular receptors (Blax *et al.* 1974).

#### References

- B. L. GASTVIX and H. RISSER, *Acta physiol. scand.* 1974, 90, 289-296.  
 L. J. PETERSON, *Physiol. Rev.* 1971, 51, 312-367.  
 A. L. JENSEN, *Acta physiol. scand.* 1972, 85, 453-463.

#### C 13

**Synthesis and Lactate Metabolism during Recovery after Maximal Exercise in Man**  
 ERHARDSEN L., O. VAADE, C. WILLIAMS, A. HELLAND and L. NILSEN, *Institute of Physiology Oslo, Norway*

Lactate can be resynthesized to glycogen in skeletal muscle as was suggested by HALL (1920) and HILL (1932), is still an open question. Studies by Cori and Cori (1929) and other investigations indicated that the liver rather than the muscle, was the site for lactate removal during recovery after exercise. Later the possibility of conversion of lactate into glycogen in the muscles has been questioned (Krebs and Woodford) due to the fact that only one of the key enzymes for a reversal of the glycolytic pathway could be demonstrated.

Studies by Erhardsen *et al.* (Unpublished results) showed that very little lactate (mmol/min) escaped from the muscles during recovery after maximal exercise. Studies (Harrison 1971) have shown a rapid fall in the lactate concentration in the blood. These studies raised the question again as to whether part of the lactate might be converted into glycogen in the muscles.

Eight young male subjects performed 3 times one min of maximal exercise to exhaustion on a bicycle ergometer with a short rest period of 4 min inbetween. After the exercise bout, the subjects rested for 30 min. Muscle biopsies (from the lateral part of triceps muscle) and blood samples were taken at rest before exercise, immediately after the third exercise bout and at the 5th, 10th, 20th and 30th min of the recovery period. The lactate concentration increased from  $0.9 \pm 0.1$  mmol/kg wet weight at rest, to  $0.9 \pm 0.1$  mmol/kg wet weight after the third exercise bout. During the recovery period the lactate concentration decreased to  $0.3 \pm 0.2$  mmol/kg wet weight. The muscle

glycogen content decreased from  $78.7 \pm 4.0$  mmol  $\text{kg}^{-1}$  wet weight (average value  $\pm$  S.E.), to  $51.0 \pm 6.7$  mmol  $\text{kg}^{-1}$  wet weight after the third exercise bout ( $p < 0.001$ ). During the recovery period glycogen content increased to  $60.2 \pm 4.2$  mmol  $\text{kg}^{-1}$  wet weight ( $p < 0.001$ ). It is concluded that the rapid synthesis of glycogen observed during the recovery period is partly explained by a resynthesis of lactate into glycogen in the muscles.

### References

- CORI C. P. and G. T. CORI *J. Biol. Chem.* 1929 **81** 389-403  
 HERMANSSEN L., O. VAAGØ, E. PRUETT and S. MATHIAS, *Unpublished results*  
 HILL, A. V. *Science* 1932, **11** 345-355  
 KARLSSON J. *Acta physiol. scand.* 1971 Suppl. 358.  
 KREBS, H. A. and M. WOOLFE *Biochem. J.* 1965 **94** 436-445  
 MEYERHOF O. *Pflügers Arch. ges. Physiol.* 1920, **182** 284-317

### C 14

#### Initial Effects of Endotoxin on Autonomic Efferent Discharges in Dogs

By M. O. HALINEN, M. O. K. HAKUMÄKI and H. S. S. SARAJÄRVI. Departments of Physiology, University of Kuopio, Kuopio and College of Veterinary Medicine, Helsinki, Finland

From the behavior of the plasma catecholamines and heart rate it has been concluded that i.v. endotoxin activates the sympathetic (Lillehei *et al.* 1964) and vagal systems (Chien *et al.* 1966). To test the validity of this indirect evidence we have explored the initial effects of endotoxin on autonomic efferent discharges in 10 mongrel dogs premedicated with *s.c.* morphine hydrochloride (1 mg/kg) and anesthetized with i.v. chloralose (100 mg/kg). Endotoxin *E. coli* (Difco) was given i.v. 1 mg/kg. In the operative period the acid-base balance was adjusted to the physiological limits. Following thoracotomy and other preparative procedures, the efferent impulse activities were recorded from the cardiac postganglionic sympathetic fibers and from vagal fibers of the left cervical vago-sympathetic trunk.

After a latency of some 30 s endotoxin elicited a sharp, partly reversible drop of blood pressure with an associated cardiac acceleration. Concurrently the cardiac sympathetic efferentation showed within a period up to 6 min a 1.5-fold increase ( $p < 0.001$ ) as compared to the control level. Thereafter the sympathetic discharges were largely normalized. The vagal efferent discharges immediately showed a slight transient reduction (N.S.) but some 2 min following the injection there was a 1.4-fold increase ( $p < 0.001$ ) in the vagal efferentation as compared to the control level. Subsequently the vagal activity too, showed a trend to restoration.

Our results are compatible with the findings of Chien *et al.* (1966) who concluded from heart rate recordings and experiments with sympathectomy that endotoxin evokes vagal activation with an antecedent transient reduction of vagal activity. More relevantly our results concur with the observations of Thümler *et al.* (1968). In dogs and cats with hemorrhagic shock, these workers found increased cardiac sympathetic efferentation during fast reduction of arterial pressure. Thus, in turn, corroborates the concept of Lillehei

) that in dogs, though not in man, endotoxin shock resembles hemorrhagic shock in its physiological characteristics.

### References

- CHANG, E. J., DELLENBACK, S., USAMI and M. I. GREGGSEN, *Am. J. Physiol.* 1966, 210  
 C., J. K. LONGERHAM, J. H. BLOCH and W. G. MAXAM, In: Schock, Ed. S. G. Hershley  
 and Company Boston 1964, Pp. 139-205  
 H. WIDENHOLM and F. KIRCHNER, *Z. Kreislaufforsch.* 1968, 58, 472-484

### C 15

Metabolic Effects of Local Cooling in Canine Subcutaneous Adipose Tissue  
 J. SOLLEVI, PAUL HJEMDAHL and BERTIL B. FREDHOLM, Department of  
 ecology Karolinska Institutet Stockholm Sweden

Subcutaneous adipose tissue is presumed to function as an insulator during cold exposure. It also contains a major store of metabolic energy. In the present study the vascular and metabolic adaptations to lowered temperature were studied in canine subcutaneous adipose tissue *in situ*.

Subcutaneous fat pad preparation (Ngai *et al.* 1966) was enclosed in a chamber perfused with blood at a controlled temperature, and its vascular resistance and release of lipolytic products were studied at 37 and 26°C. Local cooling increased resting vascular resistance 49% ( $p < 0.02$ ). Moreover the percentage decrease in vascular conductance induced by electrical nerve stimulation (4 Hz for 5 min) was enhanced by cooling (72 per cent at 26°C vs 3 per cent at 37°C,  $p < 0.05$ ). The vasoconstrictor response was also prolonged. Laboratory hyperemia was considerably delayed in time and decreased in magnitude during cooling.

During stimulation at normal temperature glycerol release increased 6-fold. After cessation of stimulation glycerol release returned to peak value within 1 min. 20-30 min later release was again normal. At 26°C glycerol release was considerably delayed, the peak release occurred 6 min after the termination of nerve stimulation and release had not returned to normal even 45 min after stimulation. The total amount of glycerol released was unchanged by cooling ( $23 \pm 6$  vs  $20 \pm 5$   $\mu\text{mol}/100$  g). The time course of FFA release was similarly affected by cooling but the total amount released was decreased (22 to  $22 \pm 8$   $\mu\text{mol}/100$  g) ( $p < 0.01$ ).

The observed increase in resting vascular resistance as well as the potentiation of electrical vasoconstriction might lead to increased thermal insulation. Lipolysis was, however, only affected to a minor degree enabling the tissue to provide adequate metabolic fuel for parts of the body.

### Reference

- H. S. ROSELI and L. WALLENGREN, Nervous regulation of blood flow in the subcutaneous adipose tissue in dogs. *Acta physiol. scand.* 1966, 68, 397-403.

# On the Functional Significance of the so-called Pneumotaxic Centre for the Respiratory Pattern Generation

By CURT VON EULER, IRJA MARTTILA, JOHN REMMERS and TERESA TRIPPENBACH  
Nobel Institute for Neurophysiology Karolinska Institutet 104 01 Stockholm

On the basis of previous results from this laboratory it was suggested by Bradley *et al.* (1975) that apnoeas would occur under conditions when the threshold for the inspiratory off-switch neurons is not reached by the input from the neurons in the solitary tract when pulmonary stretch receptor activity combines with a centrally generated inspiratory activity (CIA). Such a condition may occur as the results of 1) an increased threshold or 2) depressed CIA. The present experiments were performed to test the above hypothesis.

Cats under light pentobarbitone anaesthesia were paralyzed by gallamine and artificially ventilated 1) either proportionally to the efferent phrenic activity using a servo-respirator 2) or at a fixed rate and stroke volume. Apnoeas was obtained by restricted lesions within the medial parabrachial nucleus in rostral pons (the site of the pneumotaxic centre (Bertrand and Hugelin 1971)) and a broken volume feedback from the lungs.

It was found that the apnoeustic phrenic activity always reached higher values than the peak activity at inspiratory termination in rhythmic breathing. The apnoeustic activity of the phrenic nerve depended on  $PCO_2$  with the same sensitivity as in rhythmic breathing (Stella 1938) and with a normal dependence of the initial rate of rise of inspiratory activity on  $PCO_2$  and body temperature. In apnoeas as in intact cats both the inspiratory off-switch threshold and the CIA increased together. Consequently the duration of apnoea changed relatively little with  $CO_2$ . With increased body temperature however only CIA increased (Euler and Trippenbach 1975) with the results that apnoea's duration decreased and normal rhythmic breathing was resumed even without vagal feedback. The Hering-Breuer volume threshold for inspiratory termination was increased following the rostral pontine lesion.

Our results indicate that the so called pneumotaxic centre merely provides an excitatory threshold-lowering input to the inspiratory off switch mechanism without necessarily influencing the characteristics of either the CIA generation or the CIA-vagal interaction.

## References

- BERTRAND, P. and A. HUGELIN *J. Neurophysiol.* 1971 34 189-207  
BRADLEY, G. W. C., VON EULER, I., MARTTILA, I. and B. ROOS, *Biol. Cybernetics* 1975 in press.  
EULER, C. VON and T. TRIPPENBACH 1975 To be published  
STELLA, G., *J. Physiol. (Lond.)* 1938 93 263-275

# Isolated Gastric Glands: A New Approach for the Study of Gastric Secretion

By THOMAS BERGLINDH, Department of Physiology and Medical Biophysics University of Uppsala, Sweden

For the investigation of gastric secretion it is necessary to use both *in vitro* and *in vivo* techniques. The most useful isolated preparation so far has been the amphibian gastric

Different methods have been applied to use isolated mammalian tissue in the same report and Jensen-Chavre 1950, Kitahara *et al.* 1969) but the results have been contradictory. Isolated parietal cells have been separated from mammalian mucosa (Croft-Kinger 1969, Berglinde and Öbrink 1973) but these cells have shown little or no gastric secretagogues.

It therefore arose to separate the mucosa into functional units, i.e. into isolated glands. This was made possible by high pressure perfusion of a rabbit stomach, followed by collagenase enzyme treatment of minced pieces of the corpus mucosa. Glands obtained in this way were highly viable and had an intracellular morphology that did not differ from that seen in cells from intact animal in the electron microscope and contained essentially parietal cells and zymogen cells.

Glands showed an oxygen consumption which was constant for at least 3 hours. Due to numerous parietal cells in the gland the respiration was high ( $12.80 \pm 0.22 \mu\text{l O}_2/\text{mg glt}$  and 90 min ( $\pm 5$  E.  $n=105$ ).

Gland responses were tested for with the following common gastric secretagogues: histamine, carbachol, pentagastrin, dibutyryl cyclic AMP and aminophylline. Three different parameters were studied: a) oxygen consumption, b) degree of accumulation of the dye aminopyrine in the glandular compartment and c) stereological measurements of the parietal cell morphology.

Histamine and dibutyryl-cAMP effected all three parameters, whereas carbachol only induced the respiration and pentagastrin had no stimulatory effect. If the phosphodiesterase inhibitor aminophylline was added to histamine stimulated glands, the response was strongly potentiated.

These results suggest that histamine is the final mediator of parietal cell stimulation and that it works via the stimulation of adenylyl cyclase.

This new gastric gland preparation has also been used to study inhibitors of gastric secretion, as well as to analyse the electrolyte content in resting and stimulated glands.

It is concluded that isolated gastric glands provide a new useful tool by which the basic mechanisms of gastric acid secretion can be studied.

### References

- LINDBERG T and K. J. ÖBRINK, *Acta physiol. scand.* 1973 87: 21 A-22 A.  
 T. D. N. and E. J. INGELFINGER, *Chem. Soc.* 1969 37: 491-501.  
 JENSEN H. W. and V. JENSEN-CHAVRE, *Gastroenterology* 1950 15: 447-480.  
 JARA S. K. R. FOX and C. A. M. HOGREN, *Dig. Dis.* 1969 14: 221-238.

### C 18

**Role of Parasympathetic Bronchomotor Fibres in Airway Constriction after Solid Arterial Platelet Aggregation**

By IARLE VANG, Department of Physiology, University of Oslo, Norway

Pulmonary microembolism with platelet aggregates is a pathogenetic component in the development of the respiratory distress syndrome of the adult (Blahdell 1974). Experimentally induced platelet aggregation *in vivo* has been shown to cause a reduction in dynamic

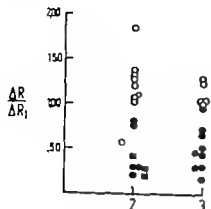


Fig. 1 Nonelastic pulmonary resistance responses to the second and third collagen infusions ( $\Delta R$ ) are given as fraction of the response to the first infusion ( $\Delta R_1$ ). The number of each individual collagen infusion is shown on the abscissa. controls,  $\bullet$  vagotomy  $\blacksquare$  atropinization.

lung compliance (dyn  $C_L$ ) and a rise in nonelastic pulmonary resistance ( $R_L$ ) as well as pulmonary vascular resistance (PVR) partly due to the release of bioactive substances from platelets (Vaage Bø and Hognestad 1975). The role of vagal reflexes in the responses has been investigated in the present experiments.

In open-chested cats during continuous positive pressure ventilation with a constant tidal volume platelet aggregation was induced by i.v. infusions of a solution of collagen fibrils. Three repeated infusions in control animals had the effect on dyn  $C_L$ ,  $R_L$  and PVR described above. The three infusions gave almost identical lung responses. Bilateral cervical vagotomy carried out before the second (or the third) collagen infusion reduced the rise in nonelastic pulmonary resistance ( $\Delta R$ ) whereas the changes in dyn  $C_L$  and PVR were unaltered as compared to the first collagen infusion. Atropinization had the same effect as vagotomy. The effects of vagotomy and atropine on  $\Delta R$  are summarized in Fig. 1.

Administration of indomethacin (20 mg/kg i.v.) to inhibit platelet aggregation abolished the responses to collagen infusions.

These observations suggest that sudden intravascular platelet aggregation induces reflex bronchoconstriction predominantly of conducting airways in which efferent parasympathetic vagal nerve fibres participate. The link between platelet aggregation and reflex bronchoconstriction is unknown.

### References

- BLAISDELL, F. W., *Arch Surg* 1974 108 44-49  
 VAAGE J. B. Bø, and J. HOGNESTAD, *Acta physiol. scand* 1975 In press.

### C 19

**Adrenoceptor Mediated Positive and Negative Feedback Control of Noradrenaline Secretion from Human Vasoconstrictor Nerves**

By L. STJÄRNE, Department of Physiology Karolinska Institutet Stockholm Sweden

Isolated superfused biopsy specimens of peripheral arteries and veins, obtained from patients undergoing surgery were used for studying factors controlling the secretion of sympathetic neurotransmitter. The tissues were preincubated with  $^3H$ -noradrenaline (NA).

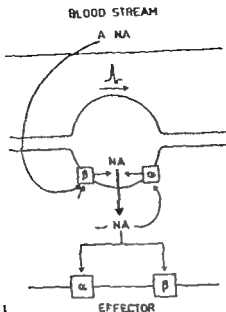


Fig 1

retion of NA was monitored by measurement of the secretion of  $^3\text{H}$ -NA evoked stimulation of the vasoconstrictor nerves, with trains of 300 shocks at 1 Hz.  $25\text{ nM}$  NA  $0.2\text{--}1\text{ }\mu\text{M}$  dose-dependently and reversibly depressed the secretion of  $^3\text{H}$ -NA.  $\alpha$ -adrenoceptor blocking drugs enhanced  $^3\text{H}$ -NA secretion. This indicates that vasoconstrictor nerves possess an  $\alpha$ -adrenoceptor function capable of sensing the local concentration of their own transmitter and of triggering restriction of further of such transmitter. These receptors appear to be set at a sensitivity level close to the high NA concentrations in the synaptic cleft, they are too insensitive to NA at the concentrations in which it normally occurs in the general circulation. It seems likely that human vasoconstrictor nerves possess an  $\alpha$ -adrenoceptor mediating autoinhibition of NA secretion (Fig. 1).

Secretion of  $^3\text{H}$ -NA appears in addition to be controlled by a  $\beta$ -adrenoceptor since the  $\beta$  agonist isoprenaline already at a concentration of  $1.6\text{ nM}$  significantly increased the secretion of  $^3\text{H}$ -NA. At  $40\text{ nM}$  the increase was  $67.7 \pm 6.2\%$ . However, during drug propranolol,  $0.1\text{--}1\text{ }\mu\text{M}$  did not significantly depress  $^3\text{H}$ -NA secretion. It appears that the secretion of sympathetic neurotransmitter in these nerves is not dependent on a  $\beta$ -adrenoceptor mediated positive feed-back loop. On the other hand, adrenaline significantly enhanced the secretion of  $^3\text{H}$ -NA, already at a concentration of  $1\text{ }\mu\text{M}$ . The effect was 'biphasic' at concentrations higher than  $40\text{ nM}$  adrenaline enhanced  $^3\text{H}$ -NA secretion even more strongly than did NA.

These results show that the secretion of  $^3\text{H}$ -NA from human vasoconstrictor nerves is enhanced by adrenaline at concentrations normally occurring as a result of stimulation of medullary secretion, and depressed by NA at concentrations normally occurring in the synaptic cleft (Fig. 1).

### Hormone Stimulated $K^+$ Transport in Isolated Rat Liver Cells

By TROND BERG and JENS-GUSTAV IVERSEN *Institute of Medical Biology Univ of Tromsø Norway*

The effects on target cells of a large group of hormones are dependent on an initial binding of the hormone to a specific receptor on the cell surface. Very soon after this interaction an increase in membrane transport of  $e.g.$  ions can be observed (reviewed by Riggs 1977). We want in this study to characterize more precisely the hormone induced changes in transport.

Unidirectional  $K^+$  fluxes were therefore measured in suspensions of isolated rat parenchymal cells (Berg *et al.* 1972) incubated with  $^{42}K$ . In vitro. By tracer exchange analysis fluxes in both directions were estimated to  $8-9 \cdot 10^{-12}$  mol/cm<sup>2</sup> sec.

Glucagon in concentrations above  $2 \cdot 10^{-8}$  M increased both influx and efflux to 160% control values. Insulin ( $100 \mu U/ml$ ) increased influx by 12-14%, whereas efflux apparently unaffected.

Using an extracellular marker  $^{51}Cr$  EDTA the intracellular level of some ions was estimated in the isolated liver cells.  $K = 172$  mmol/kg water.  $Na = 25$  mmol/kg water.  $Cl = 53$  mmol/kg water. Cellular water content: 60%.

Incubation with insulin for 1 h increased on an average intracellular concentration of  $K$  to 17 mmol/kg water. The results indicate that glucagon increases primarily the permeability of the cell membrane while insulin stimulates active  $K^+$  transport into cell.

### References

- BERG, T., D. BOMAN and P. O. SEGLEN. *Exp. Cell Res.* 1972, 72, 571-574.  
RIGGS, T. R. in *Biochemical Actions of Hormones*, Vol. 1, Ed. G. Litwack, Academic Press, N. York 1970, 157-208.

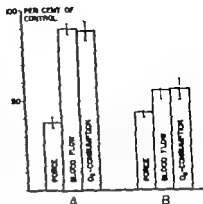
### Reduced Muscle Efficiency during Vibration-Induced Attenuation of Contractile Force in C. Soleus Muscle

By B. LJUNG, R. SIVERTSSON, M. HALLBACK and B. FOLKOW. *Department of Physiology, University of Göteborg, Sweden*

Vibrational changes in muscle length have been shown to reduce the isometric contractile force in both skeletal muscle (*e.g.* Joyce, Rack and Westbury 1969) and smooth muscle (Ljung and Sivertsson 1972). It was suggested in these reports that vibration might interfere with the contractile mechanism by causing an increased detachment of the actin-myosin cross-links. In order to further elucidate the nature of the relaxing effect exerted by longitudinal vibration, isometric force, regional blood flow and oxygen consumption were measured in the acutely denervated soleus muscle preparation of the cat (Folkow and Halicka 1968). In the control situation the steady state values for active force, blood flow and  $O_2$  consumption were measured during repeated single twitch activations of the soleus.



Fig. 1. Changes in active force, regional blood flow and  $O_2$  consumption in cat soleus muscle from control values—three repeated single twitch activations (4 Hz, 1.1 ms, 4 V) are set as 100%—to the situations illustrated in A caused by longitudinal vibrations (50 Hz, 0.5 mm) and B caused by reduction of the number of twitch motor units by lowering nerve stimulation intensity from 4 V to 0.4 V. Mean  $\pm$  S.E.,  $n=10$ .



side by suprathreshold nerve stimulation at 4 Hz, and set at 100%. Vibrations at 50 Hz with an amplitude of  $\pm 0.5$  mm were applied to the muscle tendon via the force transducer and led to an immediate decrease in twitch force. On the average the contraction amplitude was reduced by 60% but blood flow and  $O_2$  consumption were only slightly reduced from control values (Fig. 1 A). In contrast when a corresponding decrease of active muscle force is obtained by reducing the number of activated motor units (as a result of lowered stimulation intensity) a considerable reduction in blood flow and  $O_2$  consumption ensued (Fig. 1 B).

It is concluded that longitudinal vibrations interfere with the contractile process after the energy needed for the generation of force has already been expended. The results thus support the hypothesis that the mechanical movements increase the rate of cross-link formation, which would seem compatible with the finding of essentially unchanged energy consumption during vibration-induced attenuation of contractile activity.

#### References

- ROSE, C. P. M., RACK and D. R. WESTBURY. *J. Physiol. (Lond.)* 1969, 204: 461-474.  
 WILSON, J. and R. D. HALICIA. *Microvascular research* 1968, 1: 1-14.  
 ALVING, B. and E. SVENSSON. *Acta physiol. scand.* 1972, 85: 428-430.

#### C 22

Linear Distribution of U-fibres from the Postcruciate Cerebral Projection Area of the Macaque Group I Muscle Afferents

By G. GRANT, S. LANDGREN and H. SILFVÉN. *Department of Physiology, University of Umeå, and Department of Anatomy, Karolinska Institute, Stockholm, Sweden*

Group I muscle afferents from the contralateral forelimb project to area 3a near the postcruciate sulcus, Po4 (Oscarsson and Rönner 1963, 1966) and to area 4γ near the lateral sulcus of the cruciate sulcus (Silfvenius 1972) of the cat's cerebral cortex. However, Phillips, Powell and Wiesendanger (1971) did not find any responses to group I muscle afferents in the baboon's area 4γ. The cortical potential evoked in area 4γ of the cat, was of comparatively low amplitude and generally showed latency which was 1-2 ms longer

than that of the potential evoked in Ped. The question was therefore raised whether the Group I response evoked in 4 $\gamma$  was relayed via area 3a.

In the present series of experiments needle stitch lesions restricted to cortex cerebri and with a maximal diameter not exceeding 500  $\mu$ m were placed in the point near Ped when the response to electrical stimulation of the low threshold afferents of the contralateral deep radial nerve was of maximum amplitude. The degeneration of nerve terminals was studied with Fink Heimer technique after survival times of 26, 48 and 96 h.

Terminal degeneration was observed in area 4 $\gamma$  near the lateral end of the coronal sulcus as well as more medially near the location of the projection area of the Group I muscle afferents of the contralateral hind limb (Landgren and Silfvenius 1969). Terminal degeneration was also found in area 2 in the rostral bank of the lateral ansate sulcus, and in area 3b near the caudal end of the coronal sulcus. Terminal degeneration was observed in the forelimb area of SII in some of the animals.

The connection between the lesion and the degeneration areas was found to be U-fiber travelling in the white matter and reentering the cortex in a radial direction. Abundant collaterals were observed in layer III and typical terminal degeneration in layer I of the cortex. The degenerations were restricted to distinct columns with a diameter of about 1000  $\mu$ m. Up to 4 clearly separated columns were observed in area 4 $\gamma$ .

The observed connections between the projection areas of the low threshold muscle afferents in area 3a and 4 $\gamma$  may subserve the group I path to the motor cortex.

### References

- LANDGREN, S. and H. SILFVENIUS, *J. Physiol.* 1969, 200, 353-372.  
 OSCARSSON, O. and I. ROSEN, *J. Physiol.* 1963, 169, 924-945.  
 OSCARSSON, O. and I. ROSEN, *J. Physiol.* 1966, 182, 164-185.  
 PHILLIPS, C. G., T. P. S. POWELL and M. WILSENDANGER, *J. Physiol.* 1971, 217, 419-446.  
 SILFVENIUS, H. Umeå University medical dissertations, 1972, No. 4.

### C 23

#### Water Permeability in the Human Forearm during Graded Exercise

By T. PALM, S. L. NIELSEN and N. A. LASSEN, *Department of Clinical Physiology, Bispebjerg Hospital, Copenhagen, Denmark*

It is difficult to measure changes in water permeability in skeletal muscle during rhythmic exercise with plethysmographic techniques, but in animal experiments an increase in capillary filtration coefficient (CFC) with work load has been observed. Using an osmotic transient technique it is possible in the human forearm to study the water permeability without changing the prevailing hydrostatic and crystalloid osmotic pressures. The osmotic gradient is created by close intraarterial infusion of 25% human albumin solution dialyzed to crystalloid osmotic isotonicity, and the water concentration in the blood samples from the deep veins of the forearm can be determined from concentration of water marker in the arterial blood (<sup>125</sup>Iodide-albumin) and infused solution (uniodinated albumin). The principle has recently been used in dog lungs with crystalloids as osmotic driving force (Effros 1974). In the present study on normal subjects graded exercise was

performed with a hand ergometer and blood flow was measured by dye-dilution technique and strain gauge plethysmography

the amount of water that was sucked into the blood during a single passage through the vascular bed in the muscle showed a linear relation to the suction forces i.e. osmotic pressure of albumin. Knowing the relative blood flow a capillary filtration index can be calculated from the amount of water per ml blood per mm Hg times flow. CFC values were somewhat higher than found by measurements with plethysmographic technique. With increasing work load the amount of water sucked into each ml of log blood was independent of blood flow. This means that a linear relation existed between CFC and blood flow in the range from resting values to maximal dilatation. If it is noted that water permeability does not change during exercise then changes in CFC reflect changes in surface area, i.e. surface area increased in proportion to the change in flow in accordance with the concept formulated by Krogh of recruitment of capillaries increasing work load

#### References

25. R. M. J. Clin Invest. 1974 54 935-947

#### C 24

Environmental Adaptation and Multiplicity in Hemoglobins of the Flatfish Plaice and Flounder

By ROY E. WEBER, Department of Zoophysiology University of Aarhus, Denmark

The *Pleuronectes platessa* and flounder (*Platichthys flesus*) are inactive flatfish, inhabiting coastal benthic habitats. Flounders, however, occur closer inshore than plaice and thus subject to greater variation in ambient conditions. Flounders accordingly show greater tolerance to environmental temperature and salinity than plaice (Wade 1944). This report describes the oxygenational properties of the hemoglobin systems of these fish, aiming to trace the possible adaptations to environmental conditions.

The hemoglobin concentration in flounder blood is about 50 per cent higher than in plaice. Flounder hemoglobin has a higher oxygen affinity than plaice in the red cells, as well as in purified solutions. Flounder hemoglobin is moreover less sensitive to pH, both as regards oxygen affinity (Bohr effect) and oxygen capacity (Root effect).

Compared to many other fish, the ratio of ATP (a potent regulator of oxygen affinity in the blood) to hemoglobin is low in both species, amounting to about 1.5. The P<sub>50</sub>/hemoglobin ratio is however well adjusted to the fact that ATP exerts greater influence on P<sub>50</sub> (half-saturation oxygen tension) of plaice hemoglobin at low concentrations (Weber and De Witte 1975).

In both plaice and flounder the main hemoglobin components (separated by iso-electric focusing) show similar Bohr and ATP effects, and these properties moreover correspond to those of the whole hemolysates (Figure 1) lacking evidence for interaction between occurring components in vivo. In their functional similarity the multiple hemoglobins of plaice and flounder differ from those in trout, salmon and eel, which contain pH

than that of the potential evoked in Pcd. The question was therefore raised whether Group I response evoked in 4  $\gamma$  was relayed via area 3 a.

In the present series of experiments needle stitch lesions restricted to cortex ce with a maximal diameter not exceeding 500  $\mu$ m were placed in the point near Po the response to electrical stimulation of the low threshold afferents of the contralateral deep radial nerve was of maximum amplitude. The degeneration of nerve terminals studied with Fink Heimer technique after survival times of 26, 48 and 96 h.

Terminal degeneration was observed in area 4  $\gamma$  near the lateral end of the sulcus as well as more medially near the location of the projection area of the muscle afferents of the contralateral hind limb (Landgren and Silfvenius 1969). Terminal degeneration was also found in area 2 in the rostral bank of the lateral ansate sulcus area 3 b near the caudal end of the coronal sulcus. Terminal degeneration was observed in the forelimb area of SII in some of the animals.

The connection between the lesion and the degeneration areas was found to be travelling in the white matter and reentering the cortex in a radial direction. Abundant arborizations were observed in layer III and typical terminal degeneration in layer I cortex. The degenerations were restricted to distinct columns with a diameter of 1000  $\mu$ m. Up to 4 clearly separated columns were observed in area 4  $\gamma$ .

The observed connections between the projection areas of the low threshold afferents in area 3 a and 4  $\gamma$  may subserve the group I path to the motor cortex.

#### References

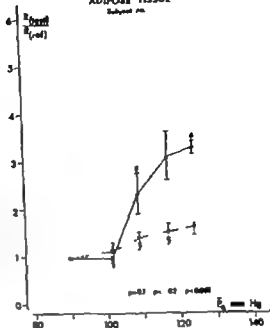
- LANDGREN, S. and H. SILFVENIUS *J Physiol* 1969 200 353-372.  
 OSCARSSON, O. and I. ROSEN *J Physiol* 1963 169 974-945.  
 OSCARSSON, O. and I. ROSEN *J Physiol* 1966 182 164-185.  
 PHILLIPS, C. G., T. P. S. POWELL and M. WEISSDÄNGER *J Physiol* 1971 277 419-446.  
 SILFVENIUS, H. Umeå University medical dissertations. 1972 No. 4.

#### C 23

#### Water Permeability in the Human Forearm during Graded Exercise

By T. PALM, S. L. NIELSEN and N. A. LASSEN *Department of Clinical Physiology, Bispebjerg Hospital, Copenhagen, Denmark*

It is difficult to measure changes in water permeability in skeletal muscle during rhythmic exercise with plethysmographic techniques, but in animal experiments an increase in capillary filtration coefficient (CFC) with work load has been observed. Using an osmotic transient technique it is possible in the human forearm to study the water permeability without changing the prevailing hydrostatic and crystalloid osmotic pressures. The osmotic gradient is created by close intraarterial infusion of 25% human albumin solution dialyzed to crystalloid osmotic isotonicity and the water concentration in the venous samples from the deep veins of the forearm can be determined from concentration of a water marker in the arterial blood ( $^{125}$ Iodide-albumin) and infused solution ( $^{125}$ Iodide-albumin). The principle has recently been used in dog lungs with crystalloids as osmotic driving force (Effros 1974). In the present study on normal subjects graded exercise



relative change in total blood flow ( $R_{tot}/R_{rest}$ ) is plotted against arterial blood pressure ( $P_a$ ). The open and the dotted line refer to experiments performed on the distal part of the filled circles and the subbroken experiments on the distal part of the p-values indicate significance of responses obtained in forearm as calculated by the randomization two independent samples. The values given are  $\pm$  S.E.

to an increase in transmural pressure was more pronounced in orthostatic active crus than in normotensive forearm (Fig. 1).

levels above the heart where venous pressure is constant autoregulation of blood flow present in a wider arterial blood pressure range in crus than in forearm. When arterial pressure increased 25 mm Hg or more in the dependent position vascular flow increased approximately 50 per cent in forearm and 300 per cent in crus.

Influence of induced ischemia on vascular resistance was investigated in cutaneous vessels of hand and foot by means of the  $^{149}\text{I}$  Antipyrine initial slope technique. Vascular flow after maximum dilation achieved after 20, 30 and 35 min of ischemia was less in foot of the hand than in vessels of the foot. It is concluded that adaptive functional properties of vessels subjected to increased blood pressure can be demonstrated in normal crura.

## C 26

Effect of Antidiuretic Hormone on the Intrarenal Distribution of Red Cells and Plasma in the Kidney

By S. NYBO RASMUSSEN Institute of Medical Physiology B University of Copenhagen, Denmark

It is not totally clarified to what extent antidiuretic hormone (ADH) affects renal vascular resistance. Transition from water diuretics to antidiuretics is believed to be accompanied by increase in renal blood flow especially in the medulla (Thurman *et al.* 1960). The purpose

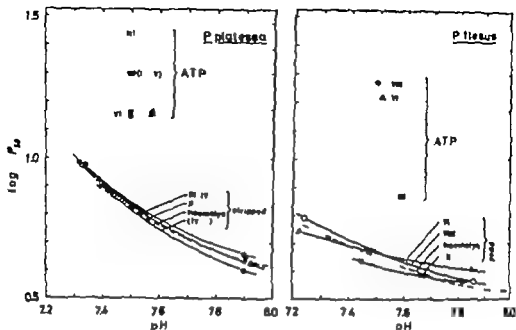


Fig. 1 Half-saturation oxygen tensions ( $P_{50}$ ) and their pH and ATP dependence of the main components of hemoglobins from plaice (left) and flounder (right) measured at 15°C in 0.1 M Tris buffers. Roman numerals represent the main components separated by (iso-electric focusing) of decreasing iso-electric point. Open symbols refer to hemoglobins that had been stripped dissolved ions, closed circles refer to hemoglobins in the presence of excess added ATP (about excess over hemoglobin). The dashed curves represent the stripped whole hemolysates.

insensitive hemoglobin components which apparently form adaptations to temporary increases in blood pH in more active fish.

The differences in hemoglobin concentration, in oxygen affinity and in pH indicate that flounder hemoglobin is better adapted than plaice hemoglobin for function under hypoxic and hypercarbic conditions. In correspondence with differences in distribution of the fish and their tolerances to environmental conditions.

#### References

- WAERDE, M. *Kleiner Meeresforschung* 1954, 10: 58-67.  
 WELSH, R. E. and J. A. M. DE WILDE, *J. comp. Physiol.* 1975. In press.

#### C 25

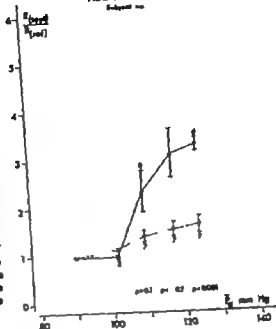
#### Vascular Resistance in Peripheral Blood Vessels at Normotension and at Local Orthohypertension in Healthy Humans

By WILLIAM F. PAASKE and OLE HENRIKSEN *Institute of Medical Physiology, University of Copenhagen, Denmark*

The influence of orthostatic pressure changes on vascular resistance in subcutaneous adipose tissue was examined at normotension in the forearm and at local orthohypertension in the crus in 5 healthy humans. Blood flow in subcutaneous tissue was measured by means of the local xenon 133 washout technique. The vasoconstrictor

ADIPOSE TISSUE  
Subject no.

change in total blood flow ( $Q_{total}/Q_{total}$ ) is plotted against total blood pressure ( $P_a$ ). The open and the dotted line refer to experiments on the distal part of the filled circles and the unbroken experiments on the distal part of the p-values indicate significance tests responses obtained in forearm is calculated by the randomization of two independent samples. The others partly I.S.E.



to an increase in transmural pressure was more pronounced in orthostatic toe cras than in normotensive forearm (Fig. 1).

In the above the heart where venous pressure is constant autoregulation of blood flow present in a wider arterial blood pressure range in cras than in forearm. When mean pressure increased 25 mm Hg or more in the dependent position vascular flow increased approximately 50 per cent in forearm and 300 per cent in cras.

The influence of induced ischemia on vascular resistance was investigated in cutaneous vessels of hand and foot by means of the  $^{141}$ I Antipyrine initial slope technique. Vascular resistance after maximum dilation achieved after 20, 30, and 35 min of ischemia was less in vessels of the hand than in vessels of the foot. It is concluded that adaptive functional changes of vessels subjected to increased blood pressure can be demonstrated in normal subjects.

## C 26

Effect of Antidiuretic Hormone on the Intrarenal Distribution of Red Cells and Plasma in the Rat

By S. NYBO RASCHUSSEN *Institute of Medical Physiology B University of Copenhagen, Denmark*

It is not totally clarified to what extent antidiuretic hormone (ADH) affects renal vascular resistance. Transition from water diuresis to antidiuretic is believed to be accompanied by a decrease in renal blood flow especially in the medulla (Thurman et al. 1960). The purpose

of this study was to evaluate the effects of ADH administration on water diuretic rat intrarenal red cell volumes, plasma volumes and haematocrits.

Water diuresis was induced in male Wistar rats by combining water loading with ethanol-thiopental anaesthesia. Infusion of arginine vasopressin  $1 \mu\text{U}/\text{min}$  gram in eight rats provoked within 15 to 30 min a rise in urine osmolality from below 200 to values ranging from 800 to 2000 mOsm per kg without significantly influencing arterial blood pressure. Another eight rats served as water diuretic controls.  $^{51}\text{Cr}$  labelled red cells,  $^{125}\text{I}$  immunoglobulin M (IgM, Mol. Wt. = 920000) were used as tracers. 60 s elapsed before i.v. injection of the tracers until clamping and freezing of the kidney. The intrarenal distribution volumes of labelled red cells and IgM in whole kidney, cortex outer and in medulla were calculated. The 60 s IgM distribution volume can be taken to represent plasma volume (Rasmussen 1975).

Red cell volume was significantly increased in all renal zones following ADH administration, whereas significant changes in regional plasma volumes could not be detected. The intravascular volume (red cell + IgM volume) was higher in cortex (and whole kidney) during ADH administration than in the water diuretic control situation. In all renal zones ADH caused substantial increments in haematocrit (red cell volume/intravascular volume). The changes in haematocrit indicate lowered blood flow in all renal zones following ADH administration. Reduction in blood flow involves decreased linear velocities of red cells with less pronounced axial accumulation of red cells and consequently higher dynamic haematocrits. The reduction in local blood flow can not be explained by changes in arterial blood pressure but must be due to increased vascular resistance. Thus, this study indicates that ADH increases total vascular resistance in the rat kidney. The data obtained do not allow any conclusions to be drawn as regards the vascular site(s) of action of the hormone.

### References

- RASMUSSEN, S. NYBO, *Pflügers Arch.* 1975. In press.  
THURAU, K., P. DEETJEN and K. KRAMER, *Pflügers Arch.* 1960, 270, 270-285.

### C 27

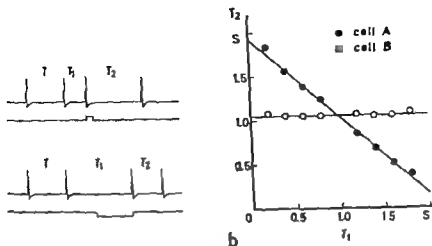
#### Two Types of Adaptation in Snail Neurones

By M. COLDING-JØRGENSEN, *Institute of Medical Physiology, Dept. A, University of Copenhagen, Denmark.*

It is generally believed that the adaptation of many excitable membranes is due to an increasing hyperpolarising current, but it is not clear whether the current is accumulated from spike to spike (impulse dependent) or is generated independently of spike formation (stimulus dependent). Two types of adaptation are therefore possible and can be separated in the following way:

Consider a membrane firing with a constant period  $T$ . The firing pattern is now interrupted by a stimulus as outlined in fig. 1a. With impulse dependent adaptation the interposed spike will increase the hyperpolarising current, prolonging the succeeding firing period, while the retardation of a spike will decrease the current, thus reducing the period.





(a) Outline of the experimental procedure. In the upper trace a spike is elicited after a stimulus at a time  $T$  smaller than the spontaneous firing period  $T$ . In the lower trace  $T$  is greater than  $T$  by hyperpolarizing stimulus. The succeeding firing period is  $T$  in both cases. (b) Relation between the firing periods  $T$  and  $T$  for two different cells. The spontaneous firing rate in both cases is between 1.0 and 1.1 s.

Each spike increases the hyperpolarizing current by the same amount, and the current then decays monoexponentially with the time constant  $\tau$ . The relation between the disturbed firing period  $T_2$  and the preceding period  $T_1$  will be almost linear for  $T/\tau < 0.25$  with a slope of  $-1/(1+T/\tau)$ . Stimulus dependent adaptation, however, an interruption will not influence the firing period.

From potentials were recorded by a single microelectrode from nerve cells in the ventral ganglion of *Hydra polymorpha*. The firing period of the cells was kept between 1.0 and 1.1 s by adjusting a bias current through the electrode. For each value of  $T_1$ ,  $T_2$  was measured as an average of 5–15 samples. It appears from fig. 1b that for cell A the relation between  $T_2$  and  $T_1$  is linear with a slope of 0.83 corresponding to a  $\tau$  of 8 s. For cell B  $T_2$  is almost constant and equal to the mean firing period of 1.05 s.

Thus, two types of adaptation can be found in small neurones, one which appears stimulus dependent, and another which does not.

## C 28

Denervation "Hypertrophy" of the Anterior Tibial Muscle after Denervation Combined with Electrical Stimulation

By I. M. KROGH, Institute of Neurophysiology, University of Copenhagen, Denmark

Denervation hypertrophy of denervated skeletal muscle of rats has been described by Kohn (1960). Already after 4 hrs the non-collagen protein content of the anterior tibial muscle was increased by 10%. Denervation hypertrophy was observed also in the hemi-

diaphragm of rats one week after unilateral phrenicotomy and attributed the stretch by innervated hemi-diaphragm. Dry and wet weight were found either increased to nearly same degree (Sola and Martin 1953, Stewart and Martin 1956, Gutmann *et al.* 1966) or gain in weight was exclusively due to water (Thomson *et al.* 1951, Thomson 1955). Diameter of denervated muscle fibres was increased by 10% (Gutmann *et al.* 1966).

24 h after denervation of anterior tibial muscle of young rats immobilized in a stretch position Kamieniecka (personal communication) found a 10–14% increase in mean fibre diameter. The aim of the study presented here was to investigate whether denervation-stretch hypertrophy was due to protein increase as after denervation alone (Gutmann 1960). In 34 rats six weeks old the right sciatic nerve was cut and the ankle fixed in plantar-flexion with plaster of Paris. After 24 h the animals were sacrificed. 10 muscles served as controls.

In sections of Epon-embedded specimens mean fibre diameter was increased by 5–10%. Wet weight was increased (+8.8%). Dry weight was increased (+3.5%) when the muscles were dried as is customary to constant weight at 95°C (at least 24 h). However, when freeze-dried under high vacuum to a final pressure of  $<10^{-6}$  mm Hg, this difference disappeared. The total amount of amino acids after acid hydrolysis was the same ( $-2.1$ ,  $p=0.4$ ). The proportion of hydroxyproline was unchanged, indicating that the ratio collagen to non-collagen protein was constant. The glycogen content was decreased ( $-5$ , less than 1% of dry weight). The lipid content was unchanged.

This study shows that hypertrophy of muscle fibres caused by denervation and constant stretch was due to increased content of water alone.

#### References

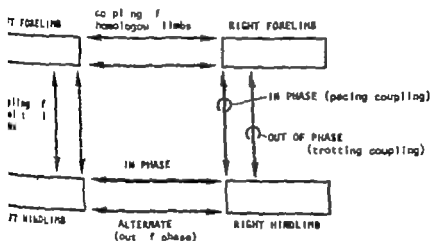
- GUTMANN, E. *Physiol. Bohemoslov.* 1960, 9, 382–389.  
 GUTMANN, E., HANIKOVA, M., HAJEK, L., KLICPERA, M., SYROVY, I. *Physiol. Bohemoslov.* 1966, 908–923.  
 SOLA, O. M., MARTIN, A. W. *Amer. J. Physiol.* 1953, 172, 324–332.  
 STEWART, D. M., MARTIN, A. W. *Amer. J. Physiol.* 1956, 186, 497–500.  
 THOMSON, J. D. *et al.* *Amer. J. Physiol.* 1951, 167, 832.  
 THOMSON, J. D., *Amer. J. Physiol.* 1955, 180, 202–204.

## DEMONSTRATIONS

## D 1

*coordination of the Limbs during Locomotion in the Cat: Basic "Programs" of Movement*  
 By I. VAN DER BURG, I. M. HALBERTSMA, F. G. A. VAN DER MECHT and  
 S. MILLER. *Department of Anatomy, Erasmus University Rotterdam, Rotterdam, The Netherlands*

In observations in cats of movements of the 4 limbs we have concluded that the basic forms of alternate locomotion, e.g. walking, trotting and swimming, and in phase motion, e.g. galloping and jumping (Miller and van der Burg 1973) result from interaction of basic programs for the phasing of flexion and extension movements of 1) the homologous limbs (hindlimbs or forelimbs) and 2) the homolateral limbs (hind- and forelimbs of the same side). See Fig. 1 A.



ALTERNATE LOCOMOTION			IN PHASE LOCOMOTION	
			= gallop	
limb		trot	1 rot gallop	jump
homologous	for limb	swimming	half bound	full bound
	hindlimb			
all	left			
	right			

(A) Diagram of the coupling of flexion and extension movements of the limbs. (B) Distribution of types of coupling in different gaits: tr gallop right transverse gallop 1. rot. gallop left rotatory gallop.

In alternate locomotion flexion and extension movements of the homologous limbs are out of phase. The homolateral limbs on both sides are coupled either approximately in phase as in pacing or out of phase as in trotting and swimming. During in phase locomotion the flexion and extension movements of the hindlimbs and to a lesser extent those of the forelimbs occur approximately in phase. In the transverse rotary and half bound gallop and occasionally in jumping the coupling of the movements of the homolateral limbs is asymmetric: on one side they are coupled in phase (pacing coupling) and on the other out of phase (trotting coupling). In the full bound and usually in jumping the homolateral limbs on both sides are coupled out of phase. (The different types of locomotor pattern are illustrated in a film.) Electromyograms from the different limbs show that transitions between the different types of coupling occur abruptly.

The hypothesis is advanced that all the characteristic patterns of locomotion in the cat result from different combinations of the "programs" of homologous and homolateral limb coupling (Fig 1B). Since these "programs" of coupling are probably organized hierarchically within the spinal cord this hypothesis carries important specifications for the functional organization of long spinal and crossed segmental connections and for the supraspinal control of locomotion.

#### Reference

- MILLER, S. and J. VAN DER BURG: The function of long propriospinal pathways in the coordination of quadrupedal stepping in the cat. In: *The Control of Posture and Locomotion* (Eds. Siera, R. J. Pearson, K. G. Smith, R. S. and Redford, J. B. Plenum Press, New York 1973) 561-578.

#### D 2

##### Applications of a Process Computer (WANG 600) in Experimental Physiology

By WILLIAM P. PAASKE and PER SEJRSSEN, Institute of Medical Physiology B, University of Copenhagen, Denmark

The equipment presently available for electronic data processing at the Institute is two process computers (WANG 600) each connected to an extended memory (WANG 618) allowing an additional 4096 program steps/512 storage registers. The input media are two high-speed electronic tape readers (FACIT 4022) and the keyboards. The output media are three tape punchers (FACIT 4070) and one plotter/printer (WANG 602). Two separate interface units (constructed by the Institute) can be connected to up to four parallel gamma spectrometers allowing on-line and/or tape-punch handling of data. A separate keyboard is connected to the interface units. These facilities are currently being used by our group in four major research projects each to be demonstrated.

1) Determination of capillary extraction in skeletal muscle and adipose tissue by the single injection, external counting method (Sejrsen 1970, Paaske and Nielsen 1973).

2) Blood flow determination from xenon-133 washout from cutaneous, subcutaneous and muscular tissues (Sejrsen 1971, Henriksen, Nielsen and Paaske 1973, Paaske, Hovind and Sejrsen 1973).

estimation of the permeability-surface area product ( $P_e S$ ) and mean body capillary flow of molecules in the entire organism  
 estimation of diffusion coefficient in agar-agar and interstitial space of tissue.

### References

- LENN O. S. L. NIELSEN and W. P. PAASKE, *Acta physiol. scand.* 1973 89 531-537  
 L. W. P. and S. L. NIELSEN, *Acta physiol. scand.* 1973 Suppl. 396: 81  
 L. W. P. H. HOVIND and PER SØRENSEN, *Scand. J. Ex. Lab. Im. et.* 1973 31 389-394  
 X. P. La. *Capillary Permeability* Alfred Benzon Symp. II C. Crone and H. A. Lassen, eds. Munksgaard, Copenhagen 1970. 256-260  
 N. P. *Measurement of Cerebral Blood Flow by Freely Diffusible Radioactive Isotopes in Copenhagen* 1971 Danish Medical Bulletin. 1971 Suppl. 18.

### D 3

**Step Kinetics of Extravascular Albumin in the Rat Kidney Studied by Means of a Step Tracer Technique**

by S. NYBO RASMUSSEN, *Institute of Medical Physiology B University of Copenhagen, Denmark*

In recent years the renal interstitium has received growing attention because of the biological significance of peritubular physical factors (i.e. hydrostatic and oncotic pressures) governing tubular fluid reabsorption (Deen *et al.* 1973 Pinter *et al.* 1974). The interstitial fluid of plasma-proteins is in a state of turnover as there is a continuous flux of protein into the pool. Proteins enter from the capillaries and leave through the lymph and finally by way of return into the capillary blood. In order to measure the rate of entrance of different plasma proteins into the renal interstitium a technique has been developed which permits to follow in time the wash-in or wash-out of radiolabelled tracer protein in the kidney.

In the rats the caudal aortae are connected to each other by means of silicone rubber tubes; the venae cavae are connected similarly. The two connecting tubes are anastomosed in a adjustable shunt placed close to the "experimental" rat, of which the left kidney is studied. This kidney may be either autoperfused or alloperfused from the heparinized donor rat. A system of electrically operated vascular clamps allows shifting the perfusion direction momentarily. Before starting a "cross perfusion" the blood of either the "experimental" or the "pump" animal is equilibrated with radiolabelled protein, e.g. albumin. When from auto- to alloperfusion or vice versa, the wash-in or wash-out of tracer in the kidney is followed continuously by external counting. The use of step input of tracer allows distinguishing between rapid intravascular and slower extravascular components in the process. The constancy of renal vascular volume during the experiment may be checked using  $^{51}\text{Cr}$  labelled red cells and  $^{125}\text{I}$  labelled protein simultaneously. The dead space of the system is extremely small and recirculation problems are minimal as opposed to the bolus injection technique.

Until now only preliminary results have been obtained but the experimental set-up has

In alternate locomotion flexion and extension movements of the homologous limbs out of phase. The homolateral limbs on both sides are coupled either approximately in phase as in pacing or out of phase as in trotting and swimming. During in phase locomotion the flexion and extension movements of the hindlimbs and to a lesser extent those of the forelimbs occur approximately in phase. In the transverse rotary and half bound, and occasionally in jumping the coupling of the movements of the homolateral limbs is asymmetric: on one side they are coupled in phase (pacing coupling) and on the other out of phase (trotting coupling). In the full bound and usually in jumping the homologous limbs on both sides are coupled out of phase. (The different types of locomotor pattern are illustrated in a film.) Electromyograms from the different limbs show that transitions between the different types of coupling occur abruptly.

The hypothesis is advanced that all the characteristic patterns of locomotion are the result of different combinations of the "programs" of homologous and homolateral coupling (Fig. 1B). Since these "programs" of coupling are probably organized within the spinal cord this hypothesis carries important specifications for the functional organization of long spinal and crossed segmental connections and for the supraspinal control of locomotion.

#### Reference

- MILLER, S. and J. VAN DER BURG: *The function of long propriospinal pathways in the coordination of quadrupedal stepping in the cat*. In: *The Control of Posture and Locomotion*. Eds. Stein and Pearson, A. G. Smith, R. S. and Redford, J. B. Plenum Press, New York 1973. 561-578.

#### D 2

#### Applications of a Process Computer (WANG 600) in Experimental Physiology

By WILLIAM P. PAASKE and PER SEJRSSEN *Institute of Medical Physiology B, University of Copenhagen, Denmark*

The equipment presently available for electronic data processing at the Institute consists of four process computers (WANG 600) each connected to an extended memory (WANG 720) allowing an additional 4096 program steps/512 storage registers. The input media are two high-speed electronic tape readers (FACIT 4022) and the keyboards. The output media are three tape-punchers (FACIT 4070) and one plotter/printer (WANG 602). Two set interface units (constructed by the Institute) can be connected to up to four parallel gamma spectrometers allowing on-line and/or tape-punch handling of data. A separate keyboard is connected to the interface units. These facilities are currently being used by our group in four major research projects, each to be demonstrated.

- 1) Determination of capillary extraction in skeletal muscle and adipose tissue by single injection, external counting method (Sejrsen 1970, Paaske and Nielsen 1973).
- 2) Blood flow determination from xenon-133 washout from cutaneous, subcutaneous and muscular tissues (Sejrsen 1971, Henniksen, Nielsen and Paaske 1973, Paaske, Henniksen and Sejrsen 1973).

ulation of the permeability-surface area product ( $P_s$ ) and mean body capillary  
 on of molecules in the entire organism.  
 termination of diffusion coefficient in agar-agar and interstitial space of tissue.

### References

- RY, O., S. L. NIELSEN and W. P. PAASAL, *Acta physiol. scand.* 1973, 89: 531-537  
 W. P. and S. L. NIELSEN, *Act. physiol. scand.* 1973, Suppl. 396: 81  
 W. P. H. HOVIND and PER SEJRSØ, *Scand. J. clin. Lab. inv.* 1973, 31: 329-344  
 P. LA, *Capillary Permeability* Alfred Benzon Symp. II C. Crone and N. A. Lassen, eds.  
 gaard, Copenhagen 1970, 256-260  
 P. Measurement of Cutaneous Blood Flow by Freely Diffusible Radioactive Isotopes  
 L. Copenhagen 1971 *Danish Medical Bulletin*, 1971, Suppl. 18.

### D 3

ge Kinetics of Extravascular Albumin in the Rat Kidney Studied by Means of a Step  
 'racer Technique

'S. NYBO RASMUSSEN, *Institute of Medical Physiology B University of Copen-  
 gen, Denmark*

: recent years the renal interstitium has received growing attention because of the  
 significance of peritubular physical factors (i.e. hydrostatic and oncotic pressures)  
 trans tubular fluid reabsorption (Deen *et al.* 1973 Pinter *et al.* 1974). The interstitial  
 f plasma-proteins is in a state of turnover as there is a continuous flux of proteins  
 in the pool. Proteins enter from the capillaries and leave through the lymph and  
 ly by way of return into the capillary blood. In order to measure the rate of  
 ation of different plasma proteins into the renal interstitium a technique has been  
 ped which permits to follow in time the wash-in or wash-out of radiolabelled tracer  
 in the kidney.

Two rats the caudal aortae are connected to each other by means of silicone rubber  
 E. vena cavae are connected similarly. The two connecting tubes are anastomosed  
 adjustable shunt placed close to the "experimental" rat, of which the left kidney is  
 study. This kidney may be either autoperfused or alloperfused from the heparinized  
 rat. A system of electrically operated vessel clamps allows shifting the perfusion  
 instantaneously. Before starting a cross perfusion the blood of either the "experi-  
 al" or the "pump animal" is equilibrated with radiolabelled protein (e.g. albumin).  
 ing from auto- to alloperfusion or vice versa, the wash-in or wash-out of tracer in the  
 ty is followed continuously by external counting. The use of step input of tracer allows  
 spacing between rapid intravascular and slower extravascular components in the  
 t. The constancy of renal vascular volume during the experiment may be checked  
 ing  $^{51}\text{Cr}$  labelled red cells and  $^{125}\text{I}$  labelled protein simultaneously. The dead space of  
 system is extremely small and recirculation problems are minimal as opposed to the  
 injection technique.

At now only preliminary results have been obtained, but the experimental set-up has

proven to work as intended and seems to offer a powerful tool in the study of circulation and fluid dynamics

### References

- DEEN W M C R ROBERTSON and D M BRÄNNLÉ *Circulation Res* 1973 33 1-8  
PINTILÄ, G B J L ATKINS and D R BELL, *Experientia* 1974 30 1043

### D 4

#### Estrous Cycle of Zinc-Deficient Female Rats

By R HARTOMA, *Department of Physiology University of Oulu Finland*

Zinc seems to be necessary for the normal pregnancy and parturition of the rat. Diminished pituitary glands and anestrus cycles have been described in severely Zn-deficient rats.

In the present investigation estrous cycles of female rats fed a diet of 12 ppm of zinc were studied.

Adult female Sprague Dawley rats were used. The diet was prepared by the laboratory of Hässle using the normal food given to the control animals. The food was available *ad libitum*. Deionised water was used. Contamination through different sources was eliminated. Special cages were prepared.

The follow-up of vaginal smears began after 6-7 weeks of diet. During the next 4 weeks there was some increase in the weight of the animals of both groups. The appearance and behaviour of the Zn-deficient rats seemed to be normal. One of the animals showed hair loss areas after 10 weeks of diet.

The control rats followed simultaneously had regular 4-day cycles. The deficient rats had a lengthened estrus. The duration increased continuously up to 7 days. One of the deficient rats had after a period of a lengthened estrus a continuous anestrus with signs of an infection. After a diet of 10 weeks normal food and water was given to this animal and it was replaced in a normal cage. Vaginal smears were controlled further on. The leucocytes soon started to decrease in the smears and disappeared completely in 7 days and a picture of proestrus followed by an estrus could be observed.

A lengthened influence of estrogen could be observed in the vaginal smears of the deficient rats. The primary change has to be detected and the level of estrogens and gonadotropins in blood should be studied.

### References

- ARGAR, J *J Nutr* 1973 103 973-981  
FOLLIS, R. H. Jr H G DAY and E. V McCOLLUM, *J Nutr* 1941 22 23-237

### D 5

#### Effect of Local Venous Stasis on Blood Flow in Human Subcutaneous Tissue

By O HENRIKSEN *Department of Nuclear Medicine NU-2033 Rigshospitalet Copenhagen Denmark*

Effect of local venous stasis on blood flow in subcutaneous tissue was investigated. <sup>133</sup>Xenon was applied in the midline on the dorsal side of a forearm in an area 3 cm long



a wide half way between elbow and wrist. A lead shield (14 cm long, 0.3 cm thick 5 cm high) was placed longitudinally on the skin in the midline of the forearm and the radioactive depot into two parts. Venous stasis was produced on one side of the forearm by another lead shield placed on the skin proximally and laterally to the depot. Blood flow in subcutaneous tissue was measured simultaneously on both sides of the shield as of the  $^{133}\text{Xe}$  washout technique.

During venous stasis on one side blood flow decreased about 40 per cent on both sides of the shield. When the tissue was infiltrated with phenolamine on the side of stasis, blood flow remained constant on this side but decreased about 40 per cent on the other side. When lidocaine was applied to the side of stasis, blood flow remained constant on both sides. When lidocaine was applied to the opposite side, blood flow decreased about 40 per cent on the side of stasis but remained constant on the other side.

Transmission due to injection and due to the induced sympathetic blockade was delimited on the side of application. This indicates that the two areas investigated are separated by a barrier at the microcirculatory level. This indicates that the increase in transmural pressure induced on the side of stasis is not transmitted to the other side.

The decrease in blood flow during venous stasis most likely is due to an increase in tension of vascular smooth muscles, as the response is blocked by lidocaine and phenolamine. Phenolamine applied to the side of stasis does not block the response on the other side, which indicates that diffusion of catecholamines from the side of stasis to the other side is not responsible. Lidocaine applied on the side of stasis blocks the response on both sides. This indicates that a vasoconstrictor stimulus elicited by venous stasis is carried to the other side by means of local nerves distributed in an area on both sides of the forearm. That application of lidocaine on the opposite side blocks the response here supports this conclusion.

Thus the vasoconstrictor response to increase in venous pressure most likely is due to a nervous mechanism involving adrenergic nerves.

## D 6

### Modeling Adaptation of Excitable Cells on Analog Computer

By M. COLDING-JØRGENSEN *Institute of Medical Physiology Dept. A, University of Copenhagen Denmark*

Activity of excitable cells can be considered to be governed by two mechanisms, one responsible for the generation of the spike, and another responsible for the adaptation of the cell by controlling the firing period. This activity is simulated on an analog computer using the two mechanisms as follows.

The spike generating mechanism is a simplification of the model presented by Hodgkin & Huxley (1952). The sodium and potassium conductances depend on the parameters measured in the first part with a fixed time constant, and their steady state values are ramp functions of the membrane potential. The model is able to display excitability and action potentials resembling those of the original model.

The firing interval is controlled by an extra potassium conductance (adaptation conductance)  $g_A$  with the time dependency

$$dg_A/dt = (G_A - g_A)/\tau$$

where the constant  $\tau$  is much greater than the other time constants of the membrane depends in general on the membrane potential but two extremes can be extracted. first  $G_A$  is zero except when the membrane is depolarised during a spike and  $g_A$  therefore only increase when a spike is elicited why the adaptation is called is dependent. In the other extreme  $G_A$  depends on the potential shift caused by an stimulus as a ramp function of the stimulus. This type of adaptation is called is dependent.

The two types are shown to behave quite differently. The impulse dependent type I to fire continuously at low frequencies and the relation between the magnitude constant stimulus and the resulting frequency can be linear over several decades. This behaves like a tonic receptor.

The behavior of the stimulus dependent type depends on the function  $G_A$ . With a slope of the ramp continuous firing is possible for large constant stimuli but not for. The relation between stimulus strength and firing frequency is nonlinear and the resembles the activity of motoneurons as presented by Granit (1972). With a large sustained firing is impossible and the membrane will stop firing after a while even for stimuli. This type behaves as a phasic receptor.

It can be concluded that the presented model with one or both of the adaptation can simulate essential properties of excitable cells.

### References

- GRANIT R. *The Sherrington Lectures XI* Liverpool 1972.  
HODGKIN A. L. and A. F. HUXLEY *J Physiol* 1952 117 500-544

### D 7

#### Membrane Noise in Receptor Neurons

By L. SJÖLIN and W. GRAMPP *Department of Physiology and Biophysics Univ of Lund Sweden*

The slowly adapting stretch receptor neurons of lobster exhibits under subthreshold conditions a membrane noise which grows with increasing depolarization and disappears to hyperpolarization beyond resting potential. This noise is resistant to 20 TEA but can be abolished by 120 nM TTX or by complete substitution of choline or Li extracellular Na. These circumstances point to the Na-system as the principal source.

For a further study of the noise generating mechanism the noise was recorded as cell noise under voltage clamp at various potential levels and analysed with respect covariance function and spectral density. From this analysis it became apparent 1) the process underlying noise generation obeys first-order kinetics and 2) that the time

the process increases from about 1 ms to about 8 ms, as the membrane potential is from its resting level to firing threshold. This identifies the noise generating as the Na-inactivating process (Hodgkin and Huxley 1957). On the other hand the noise variance with depolarization is largely due to an increasing Na-activation, facts of which remain unrevealed by the present measurements because of the bandwidth (0-250 Hz) of the recording equipment. Thus the recorded current noise from random state transitions of the Na-inactivating system operating on channels open to be opened by the activating system. In this connexion the depolarization and increase in time constant of the inactivating process indicates that the inactivation is not turned on much under subthreshold conditions. This provides for a small delay for accommodation which may be one reason for the cells non-adapting mode of support of this assumption the fact may be quoted that in the fast adapting stretch receptor neurone it has been impossible so far to establish the existence of a specific membrane noise.

In order to see whether noise generation was localized to any particular cell region TTX was applied locally to the initial segment of the axon. This resulted in a decrease of the variance as well as in a reduction of the time constant of the noise generating process against the possibility of a high density of non-accommodating Na-channels in the initial segment of the spike trigger region of the cell.

#### Reference

HODGKIN A L and A F HUXLEY *J Physiol. (Lond.)* 1952 117 500-544.

#### D 8

Determination of Pulmonary Arterial Compliance from Registrations of Oscillating Blood Flow and Pressure

by HROAR PIEMME, Institute of Medical Biology, University of Tromsø, Norway

Registration of cyclic variations of blood flow and pressure in the pulmonary artery (PA) provides the basis for calculation of input impedance of the pulmonary vascular bed. Impedance is dependent on properties of blood, on vascular dimensions and on vessel compliance. Measurement of impedance may therefore give more information of the vascular system than conventional resistance measurement. Since vascular parameters (compliance) influence vascular impedance in a rather complex way, changes in impedance give no obvious information of changes in the vascular system. It may be possible, however, to estimate vessel compliance by the use of mathematical analogs of the star system, requiring identical impedance values of the analog and the vascular system. The simplest possible analog of the pulmonary arterial system, which takes vessel elasticity into account, is a lumped compliance, here denoted "effective arterial compliance", paralleled by arterial resistance. Based on determination of PA input impedance and phase difference between pressure and flow oscillations ( $\phi$ ) at heart frequency ( $f$ ), effective arterial compliance ( $C_{eff}$ ) is calculated from:

$$C_{eff} = -\sin \phi / 2\pi f Z$$

This method was preliminarily validated in a computer model of the pulmonary arterial system of rabbits comprising vessels down to 50  $\mu\text{m}$  diameter. Arterial compliance in the computer model could be changed by simulating altered myogenic tone of the vessel walls. Input impedance of the computer model was used to calculate  $C_{\text{eff}}$  and a highly positive correlation ( $r=0.99$ ) was found between  $C_{\text{eff}}$  and total arterial compliance in the computer model.

The method was used to calculate changes in pulmonary arterial compliance during stimulation of sympathetic nerves to cat lungs. During such stimulation  $C_{\text{eff}}$  was reduced by 60% from the control value (from  $2.4$  to  $0.9 \times 10^{-3} \text{ cm}^3/\text{dyne}$   $p < 0.01$ ) which is in accordance with reported changes in pulmonary arterial compliance of dog lungs (Ingram *et al.* 1968).

At the present time the method seems to be useful for detecting and quantifying changes in compliance of the pulmonary arterial bed.

### Reference

INGRAM, R. H., J. P. SZIDON, R. SKALAK and A. F. FISHMAN, *Circ. Res.* 1968, 22, 801-815.

### D 9

#### Phenylethanolamine-N-methyl Transferase (PNMT) Activity in Sympathetic Ganglia of the Cod

By T. ABRAHAMSSON and S. NILSSON, *Department of Zoophysiology, University of Göteborg, Sweden*

The content of both adrenaline and noradrenaline in the cod spleen decreases significantly after sympathetic nerve sectioning or injection of reserpine or 6-hydroxydopamine (Abrahamsson and Nilsson 1975) which suggests that both amines are stored in the nerve fibres and thus may act as transmitter substances. Adrenaline is the dominating catecholamine in the cod spleen while in an other adrenergically innervated organ, the swimbladder muscularis mucosae noradrenaline dominates (Euler and Flänge 1961). This indicates that adrenergic fibres of the same animal have a different catecholamine dominating in the nerve fibres of different organs. There is a possibility that in the cod adrenaline synthesized in chromaffin tissue of the head kidney and released into the blood, is taken up and stored in adrenergic fibres. A difference in blood supply to the organs could explain the differences in adrenaline/noradrenaline ratio. Among other vertebrates, adrenaline has been found to be the dominating autonomic adrenergic transmitter in amphibians (Burnstock 1969).

In the present study the activity of PNMT in the head kidney and the sympathetic ganglia/splanchnic nerve was analyzed to elucidate whether a local synthesis of adrenaline from noradrenaline can take place in the sympathetic neurons of the cod *Gadus morhua*.

To analyze the enzymatic activity of PNMT the method of Axelrod (1962) partly as modified by Mazeaud (1971) was used. The synthesis of metanephrine from normetanephrine was identified by paper chromatography. The results show a PNMT activity in both the head kidney  $1.19 \pm 0.16 \text{ U/g}$  ( $n=3$ ) and in the sympathetic neurons

lg ( $n=9$ ) (mean  $\pm$  S.E.). One unit (U) equals 1 nmol of metanephrine formed per g tissue per hr. In the sympathetic neurons the differences in frequency in the sample and blank were tested statistically (t-test for paired values) least differences ( $p < 0.001$ ).

Since in the sympathetic neurons indicate a local synthesis of noradrenaline in these. This can be taken as further evidence that together with noradrenaline, is acting as transmitter substance in adrenergic organs of the cod.

### References

- N. T. and S. NILSSON, *Comp. Biochem. Physiol.* 1975. In press.  
*J. Biol. Chem.* 1962. 237. 1637-1640.  
 O., *Pharm. Rev.* 1969. 21. 247-324.  
 von and R. FÄLCK, *Gen. Comp. Endocrinol.* 1961. 7. 191-194.  
 L., Thesis, Faculty of Science, Paris 1971.

### D 18

#### Nerve Control of the Dogfish Spleen

NILSSON, S. HOLMÖREN and D. J. GROVE. Department of Zoophysiology  
 City of Göteborg, Sweden and Marine Science Laboratories, Menai Bridge

human spleen is supplied by postganglionic sympathetic fibres which are mainly cholinergic, although sympathetic cholinergic fibres have been demonstrated (see review and Widdington 1973).

There are few reports on the effects of drugs and nerve control of the fish spleen. The teleost, *Gadus morhua*, releases erythrocytes when stimulated (Nilsson and Grove 1974), while Opdyke and Opdyke (1971) concluded that the elasmobranch (*Squalus*) spleen has not developed this capacity.

Work was made to elucidate the effects of some adrenergic and cholinergic drugs and electrical stimulation on the perfused dogfish (*Scyliorhinus canicula* and *Squalus*) spleen, and on isolated spleen strips and artery strips from these species. Perfused spleen from either species contracted in response to adrenaline, norepinephrine and phenylephrine thereby releasing red blood cells at the beginning of each contraction. Isoprenaline in low doses caused small splenodilatation, which was reversed by higher doses. Isolated spleen strips contracted in response to the alpha adrenoceptor agonists and so did strips from the coeliac or hepatic artery. Isoprenaline had no effect on the spleen strips from either species but the artery strips in the concentration range  $10^{-7}$ – $10^{-6}$  M, while higher concentrations produced contraction.

Acetylcholine had very irregular effects on the perfused preparations, and the responses decreased rapidly due to desensitization of the preparation. The spleen strips from *Squalus* did not respond at all to acetylcholine, while those from *Squalus* usually

contracted weakly. The artery strips from both species however responded well to acetylcholine giving proper dose-response curves.

Reproducible effects of electrical stimulation of the splenic nerves in the perfused spleen preparation were seen in *Squalus* only. The constriction obtained during stimulation could be abolished by relatively low concentrations of phentolamine ( $10^{-7}$  M) present in the perfusion fluid while atropine (up to  $10^{-8}$  M) caused an enhancement of the nerve response. Mecamylamine ( $10^{-6}$  M) had no detectable effect on the nerve response.

It is concluded that the *Squalus* spleen is regulated primarily by adrenergic fibres. Presence of a cholinergic link in the nervous pathways to the different effectors in the spleen (vessels trabeculae capsule) is possible but could not be detected with certainty in the present work. The *Scyliorhinus* spleen may be regulated to a large extent by circulating catecholamines since the nerve supply appears to be poor. Changes in plasma concentrations of adrenaline and noradrenaline during stress is within the range where we find contraction of isolated spleen strips or artery strips occur (Mazeaud 1969).

### References

- DAVIES, H. N. and P. G. WILKINSON *Pharm. Rev.* 1973 25 373-413.  
 MAZEAUD M. C. R. *Stam. Soc. Biol.* 1969 163 2262-2266.  
 NILSSON, S. and D. J. GROVE, *Eur. J. Pharmacol.* 1974 28 135-143.  
 OPHYKE, D. F. and N. E. OPHYKE, *Amer. J. Physiol.* 1971 221 623-625.

### D 11

#### Drug Effects on Gas Production and Smooth Muscles in a Fish Swimbladder

By R. FÄNGE, S. HOLMGREN and S. NILSSON *Department of Zoophysiology University of Göteborg Sweden*

The swimbladder of the wrasse (*Ctenolabrus rupestris*) is a closed cavity which is divided into an anterior and a posterior part by a transverse diaphragm with a central hole. The swimbladder is filled by secretion of mainly oxygen from the flattened reddish gas gland in the ventral part of the anterior portion. During gas secretion the diaphragm moves backwards by movements in the smooth muscle of the mucosa, thereby enlarging the anterior or secretory part considerably. The gas is resorbed when the secretory part contracts and thereby moves the diaphragm forward and enlarges the thin, gaspermeable posterior or resorber part (Fänge 1953 1973).

Responses of strips from the anterior or posterior parts of the mucosa to different drugs were recorded as cumulative dose-response curves. The anterior part contracts in response to the adrenergic agonists adrenaline noradrenaline and phenylephrine and to acetylcholine and 5-hydroxytryptamine while isoprenaline has no effects. The order of potency of the adrenergic agonists and the fact that phentolamine produces a parallel shift of the noradrenaline dose-response curve indicates the presence of alpha adrenoceptors. The posterior part contracts in response to acetylcholine. Strips precontracted with acetylcholine relax by addition of isoprenaline adrenaline and noradrenaline and the relaxation is competitively antagonized by propranolol indicating that the effects are due to

of beta adrenoceptors. Precontracted strips are also relaxed by tryptamine.

Injection of 0.5 ml of the swimbladder gas stimulates gas secretion from the gas gland. In the presence of atropine or mecamylamine immediately after removal of the gas, the production is significantly inhibited. No significant alterations in gas secretion after removal of gas is produced by reserpine, yohimbine or 6-hydroxydopamine. However in the presence of these drugs which have not been subject to gas removal reserpine, yohimbine or 6-hydroxydopamine cause a backward movement of the diaphragm increasing the area of the anterior part.

Recent histochemistry reveals smooth and varicose adrenergic fibres in both parts of the muscularis mucosae. Yellow fluorescent cells, similar to those described in *Salmo gairdneri* (Peters 1965), which may contain 5-hydroxytryptamine, are also seen. It is concluded that a cholinergic pathway may be required for gas secretion in the anterior part. A considerable part of the nervous control of the muscularis mucosae may be adrenergic fibres, which act via alpha adrenoceptors in the anterior part and beta adrenoceptors in the posterior part.

#### References

1. G. B. PALCK and E. ROSENTHAL. *Acta physiol. scand.* 1965. 64: 119-126.  
 2. R. PALCK. *Acta physiol. scand.* 1953. 39: Suppl. 110. 1-133.  
 3. R. LA COMPAGNE. *Physiology*. Ed. L. Bolis, R. Schmidt-Nielsen and E. H. P. Madrell. 1973. 199.

#### D 12

**Method for On Line Calculation of Time Interval and Peak Amplitude Histograms**

Y. L. SJÖLIN. *Department of Physiology and Biophysics, University of Lund, Sweden*

Biological experiments are normally characterized by the accumulation of large quantities of raw data. Manual processing of these data is often tedious and time consuming and can only be performed off line. Often this may result in unnecessary repetition of the experiment.

In connection with studies of Schwann cell miniature end plate potentials (min. e.p.p.) and single action potentials of frog skeletal muscle (Birks, Katz and Miledi 1960), it was felt that a considerable effort in data processing would be saved if a digital handling system that could calculate peak amplitude or time interval histograms was constructed. The time interval histogram should include the determination of latency time, time between successive events, and the time

of the signal to be processed. In the peak amplitude mode the signal is passed on to an analog to digital converter so designed that it determines the peak value of the min. e.p.p. and converts it into an eight bit binary word. In time interval measurements the elapsed time is converted into an eight bit binary word. This word represents a certain address within a memory array (256 memory cells, each containing an eight bit word). Whenever a certain memory cell is addressed by the peak detector its content is increased by one. At the time

when the content of any cell reaches 240 the memory is disabled. Thus considering the measured peak voltage or time interval corresponds to an eight bit binary word voltage levels or time intervals containing a maximum of 240 counts can be presented in a histogram is continuously displayed by means of a step generator that sequentially addresses all the memory cells. The address signal is digital to analog converted and fed x-amplifier of a cathode ray oscilloscope. The eight bit binary word that represents content of a cell is likewise converted to an analog signal and displayed vertically on oscilloscope.

The machine utilises mainly TTL logic circuitry and the total costs amount to 150£.

### Reference

BIRKS, R. B. KATZ and R. MILEDI, *J. Physiol. (Lond.)* 1960 150, 145-168.

### D 13

#### Actions of Local Anaesthetics Applied by Microiontophoresis to Motoneurons

By I. ENGBERG, J. A. FLATMAN, K. KADZIELAWA and J. D. C. LAMBERT *Department of Physiology Aarhus University Denmark*

Several amines and adrenoceptor blockers cause hyperpolarization and increase the action potentials when applied microelectrophoretically to motoneurons (Engberg, Flatman, Kadzielawa 1974). Some adrenoceptor blockers possess a local anesthetic action on excitable tissues causing a block of the spike generation mechanism.

The classical local anesthetic agents share a similar structure to the monoamine agonists and antagonists, a terminal ring, a short intermediary chain and a terminal amino group. An investigation of the action of amethocaine, procaine and lignocaine was undertaken to characterize local anesthetic action on cat spinal motoneurons and to extend earlier investigations of Curtis and Phillis (1960).

The local anaesthetics were found to produce a rapid hyperpolarization with membrane resistance increase and increase of the spike overshoot, all of which corresponded to typical amine responses. However, towards the end of longlasting drug application spikes evoked by intracellular current injection were reduced in amplitude, and antidromic spikes were blocked. On cessation of amethocaine applications a further marked reduction and prolongation of the spike was seen together with a small membrane depolarization. The maximal action occurred up to 3 min after drug application, and a further five minutes elapsed before the spike and the membrane potential returned to normal. A similar but smaller and more rapidly reversible action was observed with procaine and lignocaine as well as with the adrenoceptor blockers pindolol and thymoxamine. Interestingly noradrenaline partially restored spikes reduced by amethocaine.

Amethocaine is more lipid soluble and more potent a local anesthetic than the other agents. This was probably reflected by its comparatively strong and longlasting action on the spike. Similarly amethocaine has a slower and more prolonged action than the other local anaesthetics at the frog node of Ranvier (Flatman 1968).

In conclusion the local anaesthetics showed two separate actions. Firstly a membrane



ization with increase of spike overshoot and membrane resistance. Secondly a resting block of the spike after the cessation of the ionophoretic current and the membrane potential to its resting level

### References

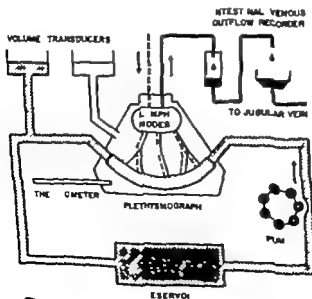
- B. and J. W. PULLIN, *J. Physiol.* 1969 153 17-34.  
 L. J. A. PLATMAN, and K. KADZELAWA, *Acta physiol. scand.* 1974, 91 3A-4A.  
 J. A. Ph.D. Thesis, University of London.

### D 14

ographic Method for Continuous Determination of Net Water Transport across  
 mal Epithelium *in vivo*

LARS JODAL, DAN-AXEL HALLBÄCK, JOAR SVANVİK and OVE LUNDGREN, *De  
 ment of Physiology University of Göteborg Sweden*

net water transport is usually measured *in vivo* by means of an unabsorbable  
 g polyethylene glycol (PEG). Since transient changes occurring within minutes  
 not detectable we have developed a method enabling such studies in  
 sized cats (see Fig). An intestinal segment is enclosed *in situ* in a perspex  
 ograph, the intestinal lumen being steadily perfused with a solution of known  
 ion from a closed perfusion system with a reservoir large enough to prevent  
 tion. Motility can be detected by direct inspection and by recording simultane-  
 ously large but opposite volume changes in the two volume transducers. If no



motility occurs the change of the perfusion volume reflects net water transport across the intestinal epithelium. A quantitative comparison of this technique with the conventional PEG-method revealed no significant difference.

The present technique does not only allow a quantitative determination of total water absorption but also an estimation of the fluid amount absorbed via the lymph. Thus control experiments show that this latter volume is recorded as a continuous increase of intestinal volume if it is assumed that net fluid exchange occurs only across the absorbing intestinal capillaries. The fluid absorbed into the lymph is "trapped" in the plethysmograph since it cannot overcome the high sealing pressure at the exit. When excluding the left volume transducer from the closed luminal perfusion system a decrease of plethysmographic volume is recorded which reflects net water absorption of fluid via the blood. The amount of fluid absorbed from the perfusion system can now be derived from its sole compliant part i.e. the intestine and fluid absorbed via the lymph remains in the plethysmograph.

#### D 15

Anaerobic Glycogen Metabolism in the Ampullarid Snail *Marisa cornuarietis*

By G. ÅKERLUND, Department of Zoology Univ. of Gothenburg Sweden

The ability of molluscs to survive anaerobic conditions has been examined by several authors. However this property varies between different groups of molluscs. Thus bivalves have been shown to withstand stressful oxygen free conditions for several days while many freshwater snails are incapable of surviving more than a few hours of anaerobiosis. Bivalvia do not accumulate lactic acid during anoxic conditions but alanine and succinic acid. The main energy pool during this period is glycogen.

However the ampullarid freshwater snail *Pila virens* which resists drought periods by aestivation has been shown to survive under laboratory conditions for 6 months in dry oxygen free environments. This snail metabolizes glycogen during aestivation at a slower rate than during anaerobiosis and accumulates lactic acid.

In my investigations I have been studying the anaerobic glycogen breakdown and lactic acid accumulation in the ampullarid *Marisa cornuarietis*. This snail is of interest because it might be used as an agent to biological control snails bearing tropical disease bilharzia. The results indicate a rapid decrease of glycogen during anaerobiosis, no accumulation of lactic acid and a tolerance of oxygen free conditions for about 16 h.

#### D 16

Correction for Changes in Counting Efficiency during Elimination of  $^{133}\text{Xe}$  from Subcutaneous Fat

By JENS BULOW and JOOP MADSEN, Medicinsk-fysiologisk institut C Copenhagen

When adipose tissue blood flow (ATBF) is determined from the rate constant for elimination of an injected  $^{133}\text{Xe}$  depot (Larsen *et al.* 1966), it is important that the subject lies immobile. Otherwise the elimination curve becomes irregular because of changed counting

activity. Consequently it has not been possible so far to determine ATBF during heavy exercise. To evade this problem the following procedure was developed.

The position of the subcutaneous  $^{125}\text{Xe}$  depot is carefully marked on the skin. At this site a radioactive  $\gamma$ -source ( $^{99\text{m}}\text{Tc}$  or  $^{57}\text{Co}$ ) is placed by means of adhesive tape. The activity is measured by a scintillation detector placed at some distance from the activity. The detector is connected to a 2 channel discriminator: one channel set for the 81 KeV  $\text{Xe}$ -peak, the other for  $^{99\text{m}}\text{Tc}$  or  $^{57}\text{Co}$ . After correction for background, resolving time, physical decay and the contribution of  $^{125}\text{Xe}$  in the  $^{99\text{m}}\text{Tc}$  (or  $^{57}\text{Co}$ ) channel variations in the  $\text{Tc}$  figures must be caused by changes in counting efficiency (geometry) affecting the  $\text{Xe}$  counts in a similar way. The  $^{125}\text{Xe}$  figures were corrected for the same determinate error as  $^{99\text{m}}\text{Tc}$  and were finally corrected for changes in counting efficiency as follows

$$^{125}\text{Xe}_{\text{corrected}} = ^{125}\text{Xe}_n \frac{^{99\text{m}}\text{Tc}_{\text{channel}}}{^{99\text{m}}\text{Tc}_n}$$

Fig. 1 demonstrates the effect of the correction procedure in a bicycle ergometer experiment. In 7 experiments on supine restless subjects, the standard deviation of the rate constant was reduced by 40-50 per cent at an average. Thus the procedure increases the reliability of proving small changes in ATBF.

#### References

REIN, O. A. N. A. LARSEN and P. QUAADE, *Acta physiol. scand.* 1966, 66, 337-343.

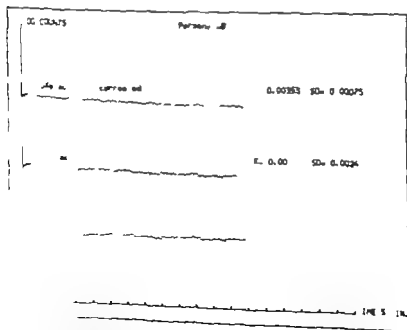


Fig. 1 Wash-out of  $^{125}\text{Xe}$  from depot in human subcutaneous fat during work on bicycle ergometer. A  $\text{Ce}$  source was placed on the skin over the  $^{125}\text{Xe}$  depot. Distance to detector: 70 cm. Registration every 20 s. Ordinate: The distance between the intercepts corresponds to one decade.

## D 17

**The Effect of Alanine on Cell Membrane Potentials in Rat Liver**

By M. FOLKE and M. PALOHEIMO. *Institute of Medical Physiology Dept. A, University of Copenhagen Jullane Mariesvej 28 DK 2100 Copenhagen Denmark*

It was recently reported that hyperpolarization of the cell membrane occurs in the perfused rat liver following the administration of various gluconeogenic substrates in large amounts (Dambach and Friedmann 1974 Folke 1974)

In our laboratory cell membrane potentials are recorded from rat livers perfused *in vitro* with a recirculating portion of oxygenated rat blood or *in vivo*

Addition of alanine (initial concentration 20 mM) to the perfusate of isolated livers causes an immediate depolarization (5–10 mV) and this is followed within 5 min by a sustained hyperpolarization (10–20 mV) *In vivo* the same response is found with concentrations of alanine an order of magnitude lower

The initial depolarization is compatible with the hypothesis that alanine may be taken up into the liver cells by a Na<sup>+</sup>-coupled transport mechanism. If this hypothesis is the most likely explanation for the subsequent hyperpolarization would be electrogenic extrusion of Na<sup>+</sup> from the cells by Na<sup>+</sup>-K<sup>+</sup> activated ATPase. In our current experiments all possibilities are investigated

**References**

- DAMBACH, G. and N. FRIEDMANN *Biochim Biophys Acta* 1974 367 366–370.  
FOLKE, M. *Acta physiol. scand.* 1974 91 18A–19A

## D 18

**The Disappearance of Hydrogen Ions from the Normal Unstimulated Stomach**

By BERTIL FRENNING *Departments of Internal Medicine and Physiology and Medical Biophysics, University of Uppsala, Sweden*

The possible reasons to the loss of hydrogen ions found on installation of hydrochloric acid into a normal unstimulated stomach has long been a matter of dispute. The effect of continuous dilution of an acid instillate by artificial non-acid secretion on the hydrogen ion loss was studied. Cat stomachs were mounted *in vivo* at the bottom of a cylindrical perspex chamber (Frenning 1972). After the preparation the stomachs were allowed to rest until they were in a resting state.

In a control period 6 ml of 170 mM hydrochloric acid was instilled into the chamber. The instillate was stirred continuously. After a 30 min period the instillate was removed. Then 6 ml of 170 mM hydrochloric acid was repeatedly instilled during 30 min periods. During these periods physiological saline was infused into the instillate by an accurate infusion pump at a rate of 1.5, 3 or 4.5 ml/30 min, respectively. The net flux of hydrogen ions during the different periods was determined. During the control period there was a mean loss of  $208 \pm 17 \mu\text{Eq}/30 \text{ min}$ . ( $\pm \text{S.E.}$ ,  $n = 5$ ). When saline was infused at a rate of 1.5 ml/30 min the loss of hydrogen ions remained at the control level ( $206 \pm 19 \mu\text{Eq}/30 \text{ min}$ ) but

When saline was infused more rapidly the loss of hydrogen ions from the instillate was reduced. When saline was infused at a rate of 3 ml/30 min the mean loss was  $140 \pm 14 \mu\text{Eq}/30$  min and when the infusion rate was 4.3 ml/30 min the mean loss was  $109 \pm 24 \mu\text{Eq}/30$  min. If the loss of hydrogen ions from the stomach was due predominantly to neutralization by an alkaline secretion—secreted either continuously or in response to exposure of the gastric mucosa to acid—little or no effect on the loss of hydrogen ions caused by infusion of saline into the instillate would be expected. The present result thus seems to indicate the occurrence of loss of hydrogen ions from the stomach by diffusion.

### Reference

Frenning, B., *Uppsala J Med Sci* 1972; Suppl 13

### D 19

#### Aspirin on the Gastro-Intestinal Permeability in the Rat

G. ÖREN, *Departments of Internal Medicine and Clinical Research, Uppsala, Sweden*

Aspirin (ASA) increases its permeability to urea to hydrochloric acid or cholinergic drugs. In man, an association between ASA and gastric reflux has been reported. The mechanism behind the effects of ASA on the gastric mucosa is not known. (Frenning 1972.) It has also been suggested that ASA may contribute to the development of gastric ulcers (Frenning 1973). The possible effects of ASA on gastric permeability were studied in rats previously anesthetized with urethane. ASA was dissolved in 100 mM HCl and given intragastrically. Intra-abdominal pH was given and animals were killed and the stomach was removed and then detected with a Geiger counter. The radioactivity of the radio-ASA dissolved in 100 mM HCl was 67.2  $\pm$  1.5 mCi/g (n = 6). No gastric reflux activity deposited in 100 mM HCl, 0.1 M NaOH or 0.1 M HCl.

### The Effect of Alanine on Cell Membrane Potentials in Rat Liver

By M. FOLKE and M. PALOHEIMO. *Institute of Medical Physiology Dept A, University of Copenhagen Jullane Mariesvej 28 DK-2100 Copenhagen Denmark*

It was recently reported that hyperpolarization of the cell membrane occurs in isolated perfused rat liver following the administration of various gluconeogenic substrates in fairly large amounts (Dambach and Friedmann 1974, Folke 1974)

In our laboratory cell membrane potentials are recorded from rat livers perfused *in vitro* with a recirculating portion of oxygenated rat blood or *in vivo*

Addition of alanine (initial concentration 20 mM) to the perfusate of isolated livers elicits an immediate depolarization (5-10 mV) and this is followed within 5 min by sustained hyperpolarization (10-20 mV) *in vivo* the same response is found with concentrations of alanine an order of magnitude lower

The initial depolarization is compatible with the hypothesis that alanine may be taken up into the liver cells by a  $\text{Na}^+$ -coupled transport mechanism. If this hypothesis is true, a likely explanation for the subsequent hyperpolarization would be electrogenic extrusion of  $\text{Na}^+$  from the cells by  $\text{Na}^+$ -K<sup>+</sup> activated ATPase. In our current experiments these possibilities are investigated

### References

- DAMBACH, G and N. FRIEDMANN. *Biochim. Biophys. Acta* 1974, 367, 366-370  
FOLKE, M. *Acta physiol. scand.* 1974, 91, 18A-19A

### The Disappearance of Hydrogen Ions from the Normal Unstimulated Stomach

By BERTIL FRENNING. *Departments of Internal Medicine and Physiology and Medical Biophysics, University of Uppsala Sweden*

The possible reasons to the loss of hydrogen ions found on installation of hydrochloric acid into a normal unstimulated stomach has long been a matter of dispute. The effect of continuous dilution of an acid instillate by artificial non-acid secretion on the hydrogen ion loss was studied. Cat stomachs were mounted *in vivo* at the bottom of a cylindrical perspex chamber (Frenning 1972). After the preparation the stomachs were allowed to rest until they were in a resting state.

In a control period 6 ml of 170 mM hydrochloric acid was instilled into the chamber. The instillate was stirred continuously. After a 30 min period the instillate was removed. Then 6 ml of 170 mM hydrochloric acid was repeatedly instilled during 30 min periods but during these periods physiological saline was infused into the instillate by an accurate infusion pump at a rate of 1.5, 3 or 4.5 ml/30 min, respectively. The net flux of hydrogen ions during the different periods was determined. During the control period there was a mean loss of  $208 \pm 17$   $\mu\text{Eq}/30$  min ( $\pm$  S.E.,  $n=5$ ). When saline was infused at a rate of 1.5 ml/30 min the loss of hydrogen ions remained at the control level ( $206 \pm 19$   $\mu\text{Eq}/30$  min) but

When saline was infused more rapidly the loss of hydrogen ions from the instillate was reduced. When saline was infused at a rate of 3 ml/30 min the mean loss was  $150 \pm 14 \mu\text{Eq}/30 \text{ min}$  and when the infusion rate was 4.5 ml/30 min the mean loss was  $109 \pm 24 \mu\text{Eq}/30 \text{ min}$ . The loss of hydrogen ions from the stomach was due predominantly to neutralization by gastric secretion—secreted either continuously or in response to exposure of the mucosa to acid—little or no effect on the loss of hydrogen ions caused by infusion of saline into the instillate would be expected. The present result thus seems to indicate the effect of loss of hydrogen ions from the stomach by diffusion.

### Reference

NO, R., *Lancet* *J Med Sci* 1972, Suppl 13.

### D 19

**Effect of Acetylsalicylic Acid on the Gastro-Intestinal Propulsion in the Rat**  
by B. FREDEN and P. G. ÖHRM, *Departments of Internal Medicine and Clinical Research II University Hospital, Uppsala, Sweden*

Exposure of the gastric mucosa to acetylsalicylic acid (ASA) increases its permeability to acid and to macromolecules. Subsequent exposure to hydrochloric acid or cholinergic stimulation causes hemorrhage and mucosal erosions. In man, an association between late intake and gastric ulcers has been suggested. The mechanism behind the effects of ASA is suggested to be an intracellular accumulation of the drug causing increased cellular pathways in the mucosa. (For references see Frenning 1972.) It has also been suggested that reflux of bile containing duodenal contents into the stomach may contribute to salicylate induced mucosal damage (Djahanagiri *et al.* 1973). The possible effect of ASA on gastric emptying and small intestinal propulsion was studied in rats previously anesthetized with permanent gastric and duodenal tubes permitting studies on unanesthetized animals. The stomach was exposed to 1.5 ml of 100 mM ASA dissolved in 100 mM HCl or to 1.5 ml of 100 mM HCl. After 30 min radioactive test meals were given. Intragastrically 1 ml of  $^{141}\text{I}$  labelled polyvinylpyrrolidone solution (PVP) was given and intraduodenally 0.2 ml  $^{141}\text{I}$  labelled PVP solution. After 15 min the animals were killed and organs placed at the cardia, the pylorus and the caecum. The specimen was removed in situ and stretched out on a perspex plate. The radioactivity was then detected with a gamma detector according to Nilsson *et al.* 1973. After 15 min the stomachs exposed to ASA dissolved in 100 mM HCl had evacuated  $79.6 \pm 5.8$  ( $\pm \text{S.E.}$ ,  $n = 5$ ) per cent of the radioactive test meal. The corresponding figures for the stomachs exposed to ASA dissolved in 100 mM NaCl was  $80.1 \pm 5.3$  ( $n = 6$ ) and for the control group  $87.6 \pm 1.6$  ( $n = 6$ ). No  $^{141}\text{I}$  could be detected in the stomach in any of the animals (i.e. no duodeno-gastric reflux occurred). The per cent of small bowel length passed by 50 per cent of the activity deposited in the duodenum was  $33.2 \pm 2.5$  in the animals exposed to ASA dissolved in 100 mM HCl,  $33.4$  in the animals exposed to ASA dissolved in 100 mM NaCl and  $67.2 \pm 1.5$  in control animals. There was no statistically significant difference between any of the

# The Effect of Alanine on Cell Membrane Potentials in Rat Liver

By M. FOLKE and M. PALOHEIMO. *Institute of Medical Physiology Dept A University of Copenhagen Juliane Mariesvej 28 DK 2100 Copenhagen Denmark*

It was recently reported that hyperpolarization of the cell membrane occurs in isolated perfused rat liver following the administration of various gluconeogenic substrates in fairly large amounts (Dambach and Friedmann 1974 Folke 1974)

In our laboratory cell membrane potentials are recorded from rat livers perfused *in vitro* with a recirculating portion of oxygenated rat blood or *in vivo*

Addition of alanine (initial concentration 20 mM) to the perfusate of isolated livers elicits an immediate depolarization (5-10 mV) and this is followed within 5 min by sustained hyperpolarization (10-20 mV) *In vivo* the same response is found with concentrations of alanine an order of magnitude lower

The initial depolarization is compatible with the hypothesis that alanine may be taken up into the liver cells by a  $\text{Na}^+$ -coupled transport mechanism. If this hypothesis is true, a likely explanation for the subsequent hyperpolarization would be electrogenic extrusion of  $\text{Na}^+$  from the cells by  $\text{Na}^+ - \text{K}^+$  activated ATPase. In our current experiments these possibilities are investigated

## References

- DAMBACH, G and N. FRIEDMANN *Biochim Biophys Acta* 1974 367 366-370.  
FOLKE, M. *Acta physiol. scand* 1974, 91 18A-19A

## D 18

# The Disappearance of Hydrogen Ions from the Normal Unstimulated Stomach

By BEATIL FRENNING *Departments of Internal Medicine and Physiology and Medical Biophysics, University of Uppsala, Sweden*

The possible reasons to the loss of hydrogen ions found on installation of hydrochloric acid into a normal unstimulated stomach has long been a matter of dispute. The effect of continuous dilution of an acid instillate by artificial non-acid secretion on the hydrogen ion loss was studied. Cat stomachs were mounted *in vivo* at the bottom of a cylindrical perspex chamber (Frenning 1972). After the preparation the stomachs were allowed to rest until they were in a resting state.

In a control period 6 ml of 170 mM hydrochloric acid was instilled into the chamber. The instillate was stirred continuously. After a 30 min period the instillate was removed. Then 6 ml of 170 mM hydrochloric acid was repeatedly instilled during 30 min periods but during these periods physiological saline was infused into the instillate by an accurate infusion pump at a rate of 1.5, 3 or 4.5 ml/30 min, respectively. The net flux of hydrogen ions during the different periods was determined. During the control period there was a mean loss of  $208 \pm 17 \mu\text{Eq}/30 \text{ min}$ . ( $\pm \text{S.E.}$ ,  $n=5$ ). When saline was infused at a rate of 1.5 ml/30 min the loss of hydrogen ions remained at the control level ( $206 \pm 19 \mu\text{Eq}/30 \text{ min}$ ) but



saline was infused more rapidly the loss of hydrogen ions from the instillate was reduced. When saline was infused at a rate of 3 ml/30 min the mean loss was  $150 \pm 14 \mu\text{Eq}/30 \text{ min}$  and when the infusion rate was 4.5 ml/30 min the mean loss was  $109 \pm 24 \mu\text{Eq}/30 \text{ min}$ . Loss of hydrogen ions from the stomach was due predominantly to neutralization by alkaline secretion—secreted either continuously or in response to exposure of the mucosa to acid—little or no effect on the loss of hydrogen ions caused by infusion into the testulane would be expected. The present result thus seems to indicate the trends of loss of hydrogen ions from the stomach by diffusion.

### References

WILKINSON, R., *Uppsala J Med Sci* 1972, Suppl 13.

### D 19

**Effect of Acetylsalicylic Acid on the Gastro-Intestinal Propulsion in the Rat**  
By B. FRENNING and P. G. ÖRTENGREN, Departments of Internal Medicine and Clinical Research II, University Hospital, Uppsala, Sweden

Exposure of the gastric mucosa to acetylsalicylic acid (ASA) increases its permeability to water and to macromolecules. Subsequent exposure to hydrochloric acid or cholinergic stimulation causes hemorrhage and mucosal erosions. In man, an association between platelet intake and gastric ulcers has been suggested. The mechanism behind the effects of ASA is suggested to be an intracellular accumulation of the drug causing increased cellular pathways in the mucosa. (For references see Frenning 1972.) It has also been suggested that reflex of bile containing duodenal contents into the stomach may contribute to salicylate induced mucosal damage (Djavanmiri *et al.* 1973). The possible effect of ASA on gastric emptying and small intestinal propulsion was studied in rats previously fitted with permanent gastric and duodenal tubes permitting studies on unanaesthetized animals. The stomach was exposed to 1.5 ml of 10 mM ASA dissolved in 100 mM HCl NaCl or to 1.5 ml of 100 mM HCl. After 30 min radioactive test meals were given. Intragastrically 1 ml of  $^{14}\text{C}$  labelled poly(vinylpyrrolidone) solution (PVP) was given and intraduodenally 0.2 ml  $^{14}\text{C}$  labelled PVP solution. After 15 min the animals were killed and stomachs placed at the cardia, the pylorus and the caecum. The specimen was removed in situ and stretched out on a perspex plate. The radioactivity was then detected with a gamma detector according to Nilsson *et al.* 1973. After 15 min the stomachs exposed to ASA dissolved in 100 mM HCl had evacuated  $79.6 \pm 5.8 (\pm 8 \text{ E.}, n = 5)$  per cent of the radioactivity in test meal. The corresponding figures for the stomachs exposed to ASA dissolved in 10 mM NaCl was  $80.1 \pm 5.3 (n = 6)$  and for the control group  $87.6 \pm 1.6 (n = 6)$ . No  $^{14}\text{C}$  could be detected in the stomach in any of the animals (i.e. no duodeno-gastric reflux occurred). The per cent of small bowel length passed by 50 per cent of the activity deposited in the duodenum was  $63.8 \pm 2.5$  in the animals exposed to ASA dissolved in 100 mM HCl,  $68.3 \pm 3.4$  in the animals exposed to ASA dissolved in 10 mM NaCl and  $67.2 \pm 1.5$  in the control animals. There was no statistically significant difference between any of the

groups. Thus no effect of ASA on the gastric evacuation and small intestinal propulsion was found. Further the result would appear to exclude contribution of bile reflux in the pathogenesis of gastric mucosal damage found after exposure of the stomach to moderate amounts of ASA.

### References

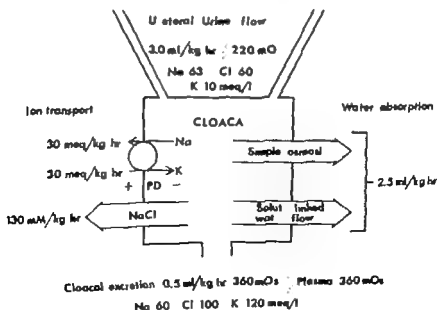
- DIAHANOURI, H. F. S., ARTANI and M. HEMMATI, *Gastroenterology* 1973 65 630-633  
 FRENHED, B., *Uppsala J med Sci* 1972, Suppl. 13  
 NILSSON, H. B., LJUNG and H. LUNDQVIST *Acta chir scand* 1973 139 716-723

### D 20

#### Cloacal Absorption in the Lizard *Agama stellio*

By I. DUVDEVANI† and E. SKADHAUG, *Department of Zoology University of Tel Aviv and Institute of Medical Physiology A University of Copenhagen*

The absorption of NaCl and water in the coprodeum of the xerophilic lizard *Agama stellio*, and the secretion of K<sup>+</sup> was evaluated by *in vivo* perfusion of the coprodeum in normally hydrated, in dehydrated and in water and salt loaded animals. The ionic and osmotic concentration of ureteral urine, of plasma, and of the cloacal contents was measured. In the normally hydrated state the ureteral urine had an osmotic urine to plasma ratio of 0.7 and it came into osmotic equilibrium in the coprodeum. Perfusion fluids of the osmolality of ureteral urine was used. In normally hydrated animals the Na and Cl absorption rates were 203 and 131  $\mu\text{eq/kg.h}$  respectively the secretion rate of K was 45  $\mu\text{eq/kg.h}$ , and the water absorption 2.4 ml/kg.h. This represents a resorption at approximately 80% of the ureterally excreted NaCl and water. In Fig. 1 the cloacal treatment of salt and water is



acted using rounded values for ureteral and cloacal excretion rates. Since the apparent osmolarity of the absorbate is only 109 mO<sub>2</sub>, the water absorption is partly due to simple osmosis, partly to a solventlinked water flow. The types of osmotic stress used all resulted changes of transport rates in directions which would serve the osmoregulatory needs of the animal.

## D 21

### Type II Fibres in Human Skeletal Muscle: Biochemical Characteristics and Distribution

By E. JANSSON, Department of Clinical Physiology Karolinska Hospital, Stockholm, Sweden

Human skeletal muscle contains two major fibre types, type I and type II, based on myosin ATPase stain (Padykula and Herman, 1955). Brooke and Kaiser (1970) have with modifications of this stain, preincubations at different acid and alkaline pHs found 3 subtypes of the type II fibres (IIA, IIB and IIC), which might indicate the existence of different kinds of myosin. The fibres also differ in oxidative and glycolytic capacities as judged from histochemical staining procedures. In order to evaluate and quantify this supposed metabolic difference single muscle fibres were dissected out, classified according to Esén et al. and analysed with regard to succinate dehydrogenase (SDH), an oxidative enzyme in Krebs cycle and phosphofructokinase (PFK) a regulatory enzyme in the glycolysis. The results (Fig. 1) reveal that the type IIB fibre has the lowest SDH activity and IIC the highest, almost as high as the activity in type I. PFK shows the opposite pattern with the highest activity in the type IIB fibre. Consequently these findings are in agreement with the previous histochemical estimate.

We have further investigated the percentage distribution of the different subgroups of type II fibres in untrained and extremely welltrained (endurance) individuals. The most interesting finding was that in the majority of the welltrained subjects no IIB fibres were found in vastus lateralis or gastrocnemius whereas the untrained had between 8 and 25%, mean 14%. It is at present not possible to tell if the extreme training has caused a change

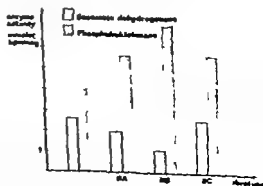


Fig. 1 Enzyme activities in individual muscle fibres. The bars represent means of determination of 3 fibres.

groups. Thus no effect of ASA on the gastric evacuation and small intestinal propulsion was found. Further the result would appear to exclude contribution of bile reflux in the pathogenesis of gastric mucosal damage found after exposure of the stomach to moderate amounts of ASA.

### References

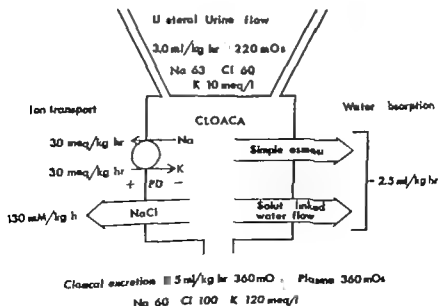
- DIAMANTOURI, B., P. S. ABTAHI and M. HEMMATI, *Gastroenterology* 1973, III 630-633.  
 FRENNING, B., *Uppsala J med. Sci.* 1972, Suppl. 13  
 NILSSON, F., B. LJUNG and H. LUNDQVIST *A la chir scand.* 1973, 139 716-723

### D 20

#### Cloacal Absorption in the Lizard *Agama stellio*

By I. DUVDEVANI † and E. SKADHAUGE. *Department of Zoology University of Tel Aviv and Institute of Medical Physiology A University of Copenhagen*

The absorption of NaCl and water in the coprodeum of the xerophilic lizard *Agama stellio*, and the secretion of K, was evaluated by *in vivo* perfusion of the coprodeum in normally hydrated, in dehydrated, and in water and salt loaded animals. The ionic and osmotic concentration of ureteral urine, of plasma, and of the cloacal contents was measured. In the normally hydrated state the ureteral urine had an osmotic urine to plasma ratio of 0.7 and it came into osmotic equilibrium in the coprodeum. Perfusion fluids of the osmolality of ureteral urine was used. In normally hydrated animals the Na and Cl absorption rates were 203 and 131  $\mu\text{eq/kg.h}$  respectively the secretion rate of K was 45  $\mu\text{eq/kg.h}$ , and the water absorption 2.4 ml/kg.h. This represents a resorption at approximately 80% of the ureterally excreted NaCl and water. In Fig 1 the cloacal treatment of salt and water is



presented using rounded values for ureteral and cloacal excretion rates. Since the apparent complexity of the absorbate is only 109 mOsm, the water absorption is partly due to simple osmosis, partly to a solute-linked water flow. The types of osmotic stress used all resulted in changes of transport rates in directions which would serve the osmoregulatory needs of the animal.

## D 21

### Type II Fibres in Human Skeletal Muscle: Biochemical Characteristics and Distribution

By E. LAMMICH, Department of Clinical Physiology Karolinska Hospital, Stockholm, Sweden

Human skeletal muscle contains two major fibre types, type I and type II, based on myofibrillar ATPase stain (Padykula and Herman, 1955). Brooke and Kaiser (1970) have with modifications of this stain, preincubations at different acid and alkaline pHs found 3 subgroups of the type II fibre (IIA, IIB and IIC), which might indicate the existence of different kinds of myosin. The fibres also differ in oxidative and glycolytic capacities as judged from histochemical staining procedures. In order to evaluate and quantify this supposed metabolic difference single muscle fibres were dissected out, classified according to Esén et al. and analysed with regard to succinate dehydrogenase (SDH), an oxidative enzyme in Krebs' cycle and phosphofructokinase (PFK) a regulatory enzyme in the glycolysis. The results (Fig. 1) reveal that the type IIB fibre has the lowest SDH activity and IIC the highest, almost as high as the activity in type I. PFK shows the opposite pattern with the highest activity in the type IIB fibre. Consequently these findings are in agreement with the previous histochemical estimate.

We have further investigated the percentage distribution of the different subgroups of the type II fibres in untrained and extremely welltrained (endurance) individuals. The most interesting finding was that in the majority of the well-trained subjects no IIB fibres were found in vastus lateralis or gastrocnemius whereas the untrained had between 8 and 25 %, mean 14 %. It is at present not possible to tell if the extreme training has caused a change

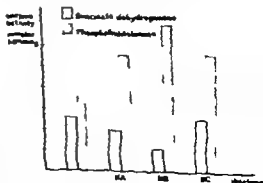


Fig. 1. Enzyme activities in individual muscle fibres. The bars represent the mean of determination of 5 fibres.

in fibre composition with the less oxidative IIB fibre converted to more oxidative IIA and a change in myosin molecule configuration, which in turn might indicate altered contraction characteristics.

### References

- BROOKE, M. H. and K. K. KAISER, *Arch. Neurol. (Chicago)* 1970, 23, 369-379.  
 ESSÉN, B., E. JANSSON, J. HENRIKSSON, A. W. TAYLOR and B. SALTIN, Metabolic characteristics of fibre types in human skeletal muscle. Under publication.  
 PADYKULA, H. A. and E. HERMAN, *J. Histochem. Cytochem.* 1955, 3, 170-195.

### D 22

#### Reactant Introduction in the Course of Assay of Oxygen Consumption Performed by Microspectrophotorespirometry

By R. HULTBORN and T. LICHTNECKERT *Institute of Neurobiology, University of Göteborg, Sweden*

A microrespirometric system for analysis of  $O_2$ -uptake of small tissue samples or single cells *in vitro* was developed by Hultborn (1974). The material to be analysed is incubated, with a medium including oxyhemoglobin, within minute ( $1 \mu\text{l}$  to  $10^{-4}$  l) chambers (cuvets) (Fig. 1) and respiration is assayed by recording the dissociation of the oxyhemoglobin due to the decreased  $O_2$ -tension caused by cellular oxygen extraction. The absorbance change of the incubate at 435 nm is recorded using a microscopespectrophotometer. The procedure has proved to be sensitive (down to  $10^{-8}$   $\mu\text{l}$   $O_2/\text{h}$ ), accurate and convenient (Hultborn 1974).

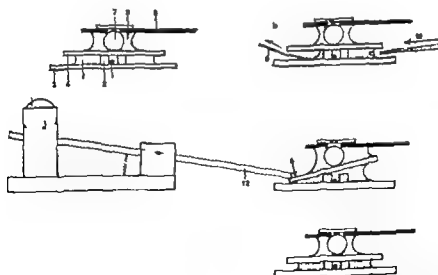


Fig. 1 (a) 1 tissue within cuvette, 2 cuvette wall, 3 plane glass slide, 4 cover glass, 5 medium surrounding cuvette, 6 steel clamp, 7 glass sphere fitted in the clamp to ascertain centric pressure, 8 immersion oil to avoid light refraction. (b) Replacement of medium outside the cuvette 9 (filter paper 10, pipette 11). (c) Taking of cover glass by a lever 12, operated by an eccentric wheel 11. (d) Assay starting from the beginning with the reactant introduced.

In many metabolic studies the absolute respiratory rate is not crucial, but rather the change of the rate on addition of a reactant, e.g. a hormone. Further it is in many cases, especially when the sample is very small, difficult to relate the  $O_2$ -uptake to some known parameter of the object (e.g. weight). It is in this case necessary to be able to relate the metabolic activity of the same sample both before and after addition of the reactant, the effect of which is to be studied.

A simple procedure fulfilling this demand has been developed for the spectrophotometric procedure. The glass covering the cuvet (see Fig.) has been extended so that it can be tilted by a lever connected to an eccentric wheel, which is moved either by hand or by a motor. Respiration under basal conditions is allowed to proceed to 50%  $O_2$ -saturation. The medium outside the cuvet is then replaced by the new one, repetitive tilting (1 sec/20 times) is commenced. Almost complete (95%) exchange of medium in the cuvet takes place and assay of respiratory rate starts from the beginning. The slopes of the curves before and after reactant introduction are compared.

In this way the problem of biological variation will be greatly diminished by comparing the results of the same sample to different reactants.

### Reference

WIK, R., *Acta physiol. scand.* 1974, Suppl. 404, 1-47

### D 23

#### Exchange of 3-O-Methylglucose in Isolated Fat Cells. Concentration dependence and effect of insulin

By L. VINTEN and J. GLIEMANN. *The Institute of Medical Physiology C University of Copenhagen, Denmark*

The self exchange of D-3-O-methylglucose was studied using a modification of the oil droplet technique described by Gliemann *et al.* (1977). It was found that the sugar equilibrates over the membrane, and that insulin is without effect on the magnitude of the distribution space at equilibrium. The disappearance of intracellular  $^{14}C$ -labelled D-3-O-methylglucose under conditions of self exchange could be described as an exponential anishing process. In the presence of insulin ( $7 \cdot 10^{-6} M$ ), the exchange rate was increased 3 times, whereas the presence of phloridzin (5 mM) reduced the exchange rate at a 3-M-glucose conc. of 50 mM about 20 times. Fig. 1 (right) shows the half time for the exchange process as a function of the sugar concentration, both in the absence and in the presence of insulin. The apparent half saturation constant for the exchange process is about 5-10 mM for both curves, whereas the transport capacity is increased about 3 times by insulin. Experiments on the inhibition by 3-O-methylglucose of the incorporation of large amounts of U- $^{14}C$ -glucose into intracellular lipids confirmed this finding. It is thus concluded, that the insulin action on the sugar transfer mechanism is to increase its transport capacity without changing its affinity to the sugar.

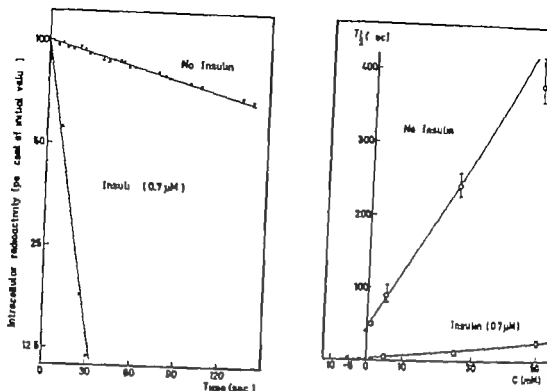


Fig. 1 The effect of insulin on exchange for 3-O-methylglucose. Left: The cells were preincubated for 30 min with 25 mM of 3-O-methylglucose and 5  $\mu$ Ci/ml of 3-O-[ $^{14}$ C]methylglucose in the absence and in the presence of insulin. The cells were centrifuged through silicone oil (specific gravity 0.97) and at zero time dispersed into a medium containing 25 mM of labelled 3-O-methylglucose. Right: The half-times of 3-O-methylglucose exchange in the absence and in the presence of insulin are plotted as a function of the 3-O-methylglucose concentration. Each point represents one efflux curve  $\pm$  SE.

### Reference

GLIEMANN, J. K. ÖSTERLUND, J. VENTEN and S. GAMMELTOFT *Biochim. Biophys. Acta* 1972, 286, 19

### D 24

**The Influence of pH on the Lactate Production by Isolated Parenchymal Rat Liver Cells**

By O. JALLING and C. OLSEN *Institute of Physiology, University of Aarhus, Denmark*

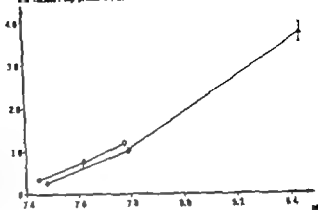
Elevated concentrations of lactate in the blood are consistently seen in connection with a rise in extracellular pH (Oliva 1970). The production as well as the removal of lactate seems to be involved. In respiratory alkalosis the normal hepatic uptake of lactate was reversed to an output; this effect was ascribed to changes in the circulation (Berry and Scheuer 1967).

The aim of the present study was to test the effect of changes in the extracellular pH,  $\text{HCO}_3^-$  and  $\text{P}_{\text{CO}_2}$  on the lactate production of isolated parenchymal liver cells.

The cells were prepared by the method of Quistorff et al. (1973). Starved rats were used. 10 mM glycerol was the substrate. 3 series of experiments were performed. The cells were incubated at 37°C in Krebs-Ringer buffer with A) HEPES 25 mM ( $n=6$ ), B)  $\text{HCO}_3^-$  25 mM and  $\text{P}_{\text{CO}_2}$  about 36, 16 and 4 mm Hg ( $n=6$ ) and C)  $\text{HCO}_3^-$  15, 25 and 35 mM and  $\text{P}_{\text{CO}_2}$  adjusted to obtain constant pH of about 7.4 ( $n=5$ ).



nm Lactate / mg protein / min



The relation between pH and lactate production by mouse liver cells. Lactatation in Krebs-Ringer buffer with 4 HEPES (circles) or with 4 HCO<sub>3</sub><sup>-</sup> (triangles).

Results from series A and B are represented in figure 1 which demonstrates a significant effect of changes in the extracellular pH on the lactate production by liver cells. Changes in the concentration of HCO<sub>3</sub><sup>-</sup> and in P<sub>CO<sub>2</sub></sub> with constant pH did not significantly influence the production of lactate which remained about 0.40 nmol lactate/mg protein/min. Results thus demonstrate enhanced output of lactate from the liver cells with rising extracellular pH, whereas changes in the concentration of bicarbonate and in the P<sub>CO<sub>2</sub></sub> with constant pH had no effect in the range tested.

Since the lactate is derived from glycerol, a production of lactate implicates an equivalent liberation of protons. It seems likely therefore that the liver participate in the compensatory processes both to respiratory and metabolic alkalosis even without changes in circulation.

### References

- RY, H. N. and J. SCHULZ, *Advances* 1967 16, 537-547  
 VA, P. B. *Am J Med* 1970 48, 209-225  
 ROSS, B. S. BONDURANT and N. G. UNWIN *Biochem Biophys Acta* 1973, 320, 503-516.

### D 25

#### Release of a Large Fraction of Oxytocin Stored in Rat Neurohypophyses by Intensive Irradiation *in vitro*

By S. DALBY, E. ENOMBERG, J. T. RUSSELL and N. A. THORN, *Institute of Medical Pharmacology, University of Copenhagen, Denmark*

Previous studies have shown that stimulation *in vitro* of vasopressin release can maximally mobilize some 10% of the total hormone content. The existence of an easily releasable fraction of vasopressin has been suggested on the basis of such experiments (Thorn 1966, Aase *et al.* 1967). To test limitations in the amounts of oxytocin that may be mobilized *in vitro* the following experiments were performed

Groups of isolated hemilobes of rat neurohypophyses were stimulated for 90 min with a 56 mM potassium concentration in the surrounding medium, and the release of oxytocin to the medium was measured by an isometric isolated rat mammary gland strip assay.

A total of 23% of the oxytocin content was released by this procedure.

When sodium was replaced by sucrose throughout the stimulation period the percentage release increased to 29%. Removal of sodium after 60 min stimulation also caused an increase in the release rate. Removal of calcium from the medium after 10 min of stimulation caused a rapid fall in the release rate. Reintroduction of calcium caused an increase of the release.

Treatment of the isolated neurohypophyses with *n*-ethyl-maleimide (NEM) (10 mM for 10 min) followed by a 90 min stimulation by 56 mM potassium caused a total release of 42% of the contents.

When after 40 min stimulation the potassium concentration of the medium was increased to 90 mM an increase in the release took place.

Neurohypophyses which were stimulated repeatedly by 56 mM potassium for 12 min followed by 9 min incubation in control medium responded with at least a fivefold increase in oxytocin release even 210 min after the start of the repetitive stimulation.

Removal of sodium and presence of NEM presumably interfere with the transport processes for  $\text{Ca}^{2+}$ . Increasing the concentration of free, intracellular  $\text{Ca}^{2+}$  which is important for release (Russell and Thorn 1975, Thorn 1974).

It is concluded that procedures which potentiate the initial processes in stimulus-secretion coupling may increase the amount of hormone that can be mobilized. Limitations in release may be more dependent on limitations in the activation processes than on maturity or localization of the hormone containing granules within the nerve endings.

## References

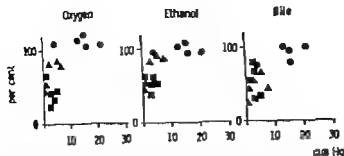
- RUSSELL, J. T. and N. A. THORN *Acta physiol. scand.* 1975 (in press).  
 SACTEL, H. L., SHARE, J., ORNCHAK, and A. CARL, *Endocrinology* 1967 81 735-770.  
 THORN, N. A. *Acta endocr. (Kbh.)* 1966, 53 644-654.  
 THORN, N. A., Role of calcium in secretory processes. In *Secretory mechanisms of lactating glands. The Alfred Benzon Symposium VII* (eds. N. A. Thorn and O. H. Petersen). Pp. 305-316. Munksgaard, Copenhagen, 1974.

## D 26

### Hepatosplanchnic Hemodynamics and Liver Function during Graded Hypoxia

By N. KRARUP, J. A. LARSEN and A. MUNCK *Institute of Physiology, University of Aarhus, Denmark*

The hepatosplanchnic vascular response to hypoxia has not yet been definitely established (Greenway and Stark 1971). Further, a critical limit of  $\text{pO}_2$  in hepatic venous blood above which a normal liver function is maintained, is not available from the literature. It was therefore of interest to study the effect of hypoxic hypoxia on the hepatosplanchnic hemodynamics and liver function. 15 chloralose anesthetized, curarized cats were ventilated with atmospheric air to a  $\text{paco}_2$  of 40 mm Hg by means of a Harvard respirator. Following



1. The hepatic oxygen consumption rate, splanchnic ethanol elimination rate and the bile flow rate as per cent of the control levels, plotted against the hepatic venous  $p_{O_2}$  during hypoxemia induced by mixtures of nitrogen and oxygen 10% (■), 12.5% (▲) and 15% (●).

1 minutes control period the inspiratory gas was altered to mixtures of nitrogen and 12.5 or 15% oxygen for a further 60 minutes period. 5 experiments were performed with each mixture of gas. The resulting hypoxemia was followed by immediate increases in arterial blood pressure which, however, returned towards control levels during the next 10 minutes, when the inspiratory gases contained 15 and 12.5% oxygen. Inspiration of 10% oxygen caused a steady decrease in arterial blood pressure. Except for a 70% increase in gastro-intestinal conductance, when the cats were inspiring 10% oxygen, no changes in conductances in the gastrointestinal area, the hepatic artery or in the intrahepatic pressure vessels occurred. The splanchnic elimination rate of ethanol, the hepatic oxygen consumption and the bile flow were all affected by the hypoxemia, but, as is apparent from Fig. 1 only when the hepatic venous  $p_{O_2}$  became lower than 10 mm Hg. This indicates that the intrahepatic perfusion is homogeneously distributed, as an uneven distribution of blood flow in the exchange vessels in the liver might result in functional disturbances at higher values of hepatic venous  $p_{O_2}$ . The finding of a gradual decrease in liver function in perfused pig livers when hepatic venous  $p_{O_2}$  was lowered from 35 mm Hg (Raboult et al. 1974) may be explained by an uneven perfusion in this preparation.

### References

- EDWARDS, C. V. and R. STABE, Hepatic vascular bed. *Physiol. Rev.* 1971. 51: 23-65.  
 ANDERSON, A. F. V., HANSEN, S., KENNEDY, N., TROSTEL, K., TONNENBERG and K. WOLLER, The effect of hypoxia on the function of the isolated perfused pig liver. *Digestion*. 1974. 10: 375-76.

### D 27

#### Cyclic AMP Content and Phosphodiesterase Activity in Rat Corpora Lutea of Different Ages

By I. KHAN, S. ROBERTO, H. HERLITZ and K. AHRÉN. *Department of Physiology, University of Göteborg, Sweden*

Many experimental findings favour the hypothesis that the stimulatory action of LH on progesterone synthesis by the corpus luteum (CL) is mediated by cyclic AMP (cAMP).

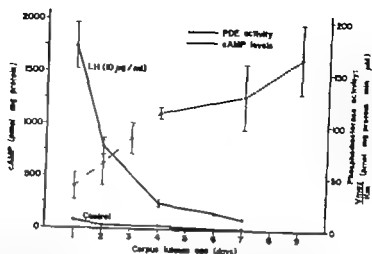


Fig. 1 cAMP and PDE activity in rat corpora lutea of different ages. Corpora lutea (CL) are isolated 1-9 days after ovulation from ovaries of PMSG-injected rats (see text). In one experiment cAMP contents were determined. CL were incubated with and without LH (10 µg/ml) for 15 min and analysed for cAMP content as described earlier (Herlitz *et al.* 1974). Mean  $\pm$  S.E. are shown in the figure (Fig. 1).

In separate experiments PDE activities were determined. CL were homogenized in Tris buffer (20 mM Tris, 6 mM MgCl<sub>2</sub>·CO<sub>3</sub>, pH 7.5) and centrifuged ( $\times 2000$  g for 10 min). Aliquots of the supernatant were mixed with equal volumes of  $^{32}$ P-cAMP (5 different concentrations between 0.1–0.01 µM) and incubated for 15, 30 and 45 min at 37°C. Degradation rates of  $^{32}$ P-cAMP were estimated according to Pösch (1971). PDE-activity ( $V_{max}/K_m$ ) was determined: a Lineweaver Burk plot. Mean  $\pm$  S.E. for 6 determinations are shown in the figure.

Marsh (1970) studied the effects of LH on the adenylate cyclase (AC) and the phosphodiesterase (PDE) in the bovine CL, and concluded that the effect of LH on progesterone production was due to stimulation of AC and not due to inhibition of PDE. It has, in addition, been shown in some species that stimulation of progesterone synthesis by LH is less effective towards the end of the CL phase. Stansfield *et al.* (1971) reported that bovine CL at the stage of regression had an increased PDE activity compared to CL collected earlier during the estrous cycle. These authors investigated, however only the enzyme with the high  $K_m$  value.

In our laboratory studies have been undertaken to follow the sensitivity to LH with respect to cAMP formation during the aging of the rat CL (Herlitz *et al.* 1974). In these studies, a "PMSG-model" has been used where pregnant mares serum gonadotropin (PMSG) has been injected to 30 day old immature rats to cause ovulation and formation of CL early in the morning of day 33. Very young CL (1 day old) show a pronounced sensitivity to LH when cAMP is studied while older CL (7 day old) show a markedly decreased response. Also the control values decreased with increasing CL age.

In the present study the above mentioned observation was correlated to the PDE activity of CL. The PDE activity in homogenates of CL of different ages were determined according to Pösch (1971) using  $^{32}$ P-labelled cAMP. No differences in activities were seen between CL of various ages when the enzyme with the high  $K_m$  (100–250 µM) was measured, but the activity of the low  $K_m$  enzyme form (0.1–0.2 µM), which seems to be more closely correlated to the physiological intracellular cAMP concentrations in CL, steadily increased with increasing age of CL (Fig. 1). The physiological meaning of this enhanced degradation

of cAMP with increasing age of the CL is not clear but it might be involved in the termination of the luteotropic effect of LH. Studies are now in progress to elucidate whether the increased PDE activity alone can explain the decreased response to LH which is seen with increasing age of CL when cAMP contents are determined (Fig. 1).

### References

- HEX, H. L. HANSEN, S. ROSSIGNOL and K. AARAS, *Acta endoc.* (Kbh.) 1974, 77 737-752.  
 KILB, J., *Am. N.Y. Acad. Sci.* 1970, 180, 416-423.  
 KILB, J. and SCHULZBERG, *Arch. Pharmac.* 1971, 268, 272-279.  
 JENSEN, D. A., J. B. HODGE and G. H. WILKINSON, *Biochem. Biophys. Acta* (Amst.) 1971, 227 413-421.

### D 28

#### Prostaglandin Formation Participates in the Control of Tone and Contractility in the Bovine Sphincter Muscle

By LARS GUSTAFSSON and PER HEDQVIST *Department of Physiology Karolinska Institute, Stockholm, Sweden*

The iris has the enzyme system necessary for prostaglandin (PG) formation (van Dorp *et al.* 1967), and PG release from iris tissue has been noted (Posner 1973). In a previous communication we have reported that exogenous PGs enhance contraction responses to orthoptic nerve stimulation whereas PG synthesis inhibitors substantially reduce tone and contraction responses in the sphincter muscle of the bovine iris (Gustafsson *et al.* 1973). In the present study we wish to report on the effects of the PG precursor arachidonic acid (TEA) on smooth muscle tone and contractility in the bovine iris sphincter muscle.

Strips of the sphincter muscle were mounted in an organ bath with Tyrode at 37°C and aerated with 5% CO<sub>2</sub> in O<sub>2</sub>. Transmural stimuli (5-15 biphasic pulses, 3 Hz, 1 ms pulse duration, supramaximal voltage) were delivered by Grass S88 stimulator through platinum electrodes along the wall of the bath at 1 min intervals.

TEA 3 µg/ml enhanced the contraction responses to transmural stimulation in 4 of 5 experiments. At 6 µg/ml it potentiated the responses in 6 of 6 experiments (p < 0.01). The responses increased over a period of approximately 10 min and then remained elevated until the preparation was washed. With the higher dose an increase in tone of the preparation was also seen. Prior administration of the PG synthesis inhibitor indomethacin 1.5 µg/ml, abolished the effects of TEA on tone and contraction responses while leaving the enhancing effect of PGE totally unaffected (Fig. 1).



Fig. 1. Response of the bovine iris sphincter muscle to transmural stimulation (3 Hz, 15 pulses at 1 ms intervals, 1 ms duration). Wash at dots. IND - indomethacin, ARACH - arachidonic acid, PGE<sub>2</sub> - prostaglandin E<sub>2</sub>.

It is suggested that the stimulant effect of TEA is mediated by newly formed PG rather than by TEA itself. The results support the view that locally formed PGs may serve the function of controlling cholinergic neuroeffector transmission and muscular tone in the sphincter muscle of the bovine iris.

### References

- DORF, D. A. VAN, G. H. JOUVENAZ and C. B. STRUIJK, *Biochim. Biophys. Acta* 1967 137 396-399.  
 GUSTAFSSON, L. P. HEDQVIST and H. LAGERCRANTZ, *Acta physiol. scand.* 1973 Suppl. 396, 106.  
 POWERS, J. *Brit. J. Pharmacol.* 1973 49 415-427.

### D 29

#### Total and Regional Oxygen Uptakes, Blood Flows and Arterial Pressures during Exercise with Smaller and Greater Muscle Groups

By N. H. SECHER, J. P. CLAUSEN, K. KLAUSEN, I. NOER and J. TRAP-JENSEN. *Laboratory for Theory of Gymnastics August Krogh Institute University of Copenhagen, and the Department of Clinical Physiology Frederiksberg Hospital Copenhagen, Denmark*

During dynamic exercise performed with the arms and the legs simultaneously the increase in oxygen uptake ( $\Delta \dot{V}O_2$ ) is lower than  $\Delta \dot{V}O_2$  for arm-exercise plus  $\Delta \dot{V}O_2$  for leg exercise measured when the arms and the legs perform their shares of the total work load separately. This could reflect either that  $\Delta \dot{V}O_2$  in tissues not in exercising arms or legs makes up a considerable part of total  $\Delta \dot{V}O_2$  during both types of exercise or that exercise with one muscle group may influence the oxygen supply to another active muscle group.

In the present study including three young healthy subjects,  $\dot{V}O_{2L}$ , cardiac output and leg blood flow (indicator dilution methods), axillary and femoral arteriovenous oxygen differences ( $AVDO_2$ ) and intraarterial blood pressures were measured during upright exercise on bicycle ergometer performed with and without concomitant arm-cranking as well as during arm-cranking alone. Arms and legs performed the same mechanical loads during all types of exercise, but arm-loads were on average 40% of leg loads. It was found that  $\dot{V}O_2$  in the exercising legs (leg blood flow times femoral  $AVDO_2$ ) was practically the same whether the legs exercised alone or in combination with arm exercise (2.5 l  $O_2$ /min. vs. 1.89 l  $O_2$ /min). Furthermore the difference between pulmonary  $\dot{V}O_2$  during combined arm- and leg exercise and  $\dot{V}O_2$  in the active legs (3.46 l  $O_2$ /min. - 1.89 l  $O_2$ /min. = 1.57 l  $O_2$ /min) was essentially the same as  $\dot{V}O_2$  during isolated arm-exercise (1.52 l  $O_2$ /min.). This suggests unimpaired  $O_2$  supply to both arms and legs during combined exercise. Nonetheless leg blood flow was reduced by 13% when arm-exercise was superimposed. Arterial mean blood pressure was unchanged or slightly increased (113 mm Hg vs. 117 mm Hg) during combined exercise as compared to leg exercise alone. Thus addition of arm-exercise caused an increased vascular resistance in the exercising legs. The main reason for this is probably an increased sympathetic vasoconstriction in the skin in proportion to the increase in relative work load. HR increased from 174 to 192 beats per min. when arm-exercise was added to leg exercise.

## D 30

## Cortical Magnification Factor in the Foveal Region of Vervet Monkey

By C. GULD. *Institute of Neurophysiology, University of Copenhagen, Denmark*

A quadrant of the retina projects to striate area on the occipital lobe, the projection is described by the magnification factor  $M$  mm/degree i.e. the distance in mm on cortex corresponding to 1° of visual field.  $M$  falls with increasing angle of visual field, is constant along a semicircle and describes a regular projection from center of fovea to the border of visual field (Daniel and Whitteridge 1961). Since later findings (Dow and Gouras 1973) indicate that center of fovea is represented some distance from the border of striate area, we examined the projection of fovea,  $\theta^\circ$  from center of fovea by recording summated potentials at known locations on cortex by monochromatic small-spot flashes at known locations on retina.  $M$  was calculated for all neighbouring pairs of positions and related to  $\theta$  as a function of semicircle and angle of meridian in visual field.

Between semicircles  $0.4^\circ$  and  $0.9^\circ$   $M$  was the same ( $P > 0.5$ ), i.e. constant along semicircles also in the foveal area.  $1/M$  as function of distances  $\theta^\circ$  from center of fovea gave the mean line  $1/M = 0.065 - 0.074 \theta$  up to  $4^\circ$  from fovea ( $N = 273$ ,  $S_{yx} = 0.061$ ,  $ME_s = 0.03$ ,  $r = 0.72$ ,  $P = 0.001$ ). With  $1/M = a + b\theta$  the area on cortex is  $A = (\pi/b^2) [\ln(1 - b\theta/a) - a/b]$  mm<sup>2</sup> ( $0 - 10^\circ$ ). For  $\theta = 0.85^\circ$   $A = 106$  mm<sup>2</sup> compared with 113 mm<sup>2</sup> calculated from corresponding coordinates on cortex.

The model shows that retina also in the foveal area is mapped regularly on cortex. Thus center of fovea is represented at the border of striate cortex, in this monkey about 3 mm posterior to the end of lunate sulcus and 3 mm above the border of occipital sulcus, agreement with the extent of striate cortex on histological preparations.

From Covey and Rolfs (1974) data on the cortical magnification factor in man, the mean  $1/M = 0.073 + 0.053\theta$  is valid up to  $15^\circ$  ( $N = 7$ ,  $S_y = -0.018$ ,  $ME_s = 0.0014$ ,  $r = 0.998$ ,  $P = 0.001$ ). Thus the magnification factor of monkey is similar to that of man at the border of fovea, but it falls more steeply with increasing visual angle in monkey than in man due to the smaller total visual area.

## References

- DAVEY, A. and ROLFS, E. T. *Exp Brain Res* 1974 21 447-454.  
 DANIEL, P. M. and WHITTERIDGE, D. *J Physiol* 1961 139 303-321.  
 DOW, B. M. and GOURAS, P. *J Neurophysiol* 1973 36 80-100.

## D 31

## Hyperpolarization of the Cell Membrane in Rat Liver after Partial Hepatectomy

By M. PALOMERINO and M. FORKE. *Institute of Medical Physiology, Dept. A, University of Copenhagen, Juhane Mortensvej 28, DK-2100 Copenhagen, Denmark*

Numerous reports in the literature indicate that rat liver has a remarkable capacity for regeneration or compensatory hyperplasia. After a two thirds hepatectomy increased DNA

It is suggested that the stimulant effect of TEA is mediated by newly formed PG rather than by TEA itself. The results support the view that locally formed PGs may serve the function of controlling cholinergic neuroeffector transmission and muscular tone in the sphincter muscle of the bovine iris.

### References

- DORF, D. A. VAN, G. H. JOUVENAZ and C. B. STRUICK, *Biochim. Biophys. Acta* 1967 137 396-399.  
 GUSTAFSSON, L., P. HEDQVIST and H. LAGERCRANTZ, *Acta physiol. scand.* 1973 Suppl. 396, 106.  
 POINIER, J. *Brit. J. Pharmacol.* 1973. 49 415-427.

### D 29

#### Total and Regional Oxygen Uptakes, Blood Flows and Arterial Pressures during Exercise with Smaller and Greater Muscle Groups

By N. H. SECHER, J. P. CLAUSEN, K. KLAUSEN, I. NOER and J. TRAP-JENSEN. *Laboratory for Theory of Gymnastics August Krogh Institute University of Copenhagen, and the Department of Clinical Physiology Frederiksberg Hospital, Copenhagen Denmark*

During dynamic exercise performed with the arms and the legs simultaneously the increase in oxygen uptake ( $\Delta \dot{V}O_2$ ) is lower than  $\Delta \dot{V}O_2$  for arm-exercise plus  $\Delta \dot{V}O_2$  for leg exercise measured when the arms and the legs perform their shares of the total work load separately. This could reflect either that  $\Delta \dot{V}O_2$  in tissues not in exercising arms or legs makes up a considerable part of total  $\Delta \dot{V}O_2$  during both types of exercise or that exercise with one muscle group may influence the oxygen supply to another active muscle group.

In the present study including three young healthy subjects,  $\dot{V}O_{2a}$ , cardiac output and leg blood flow (indicator dilution methods), axillary and femoral arteriovenous oxygen differences (AVDO<sub>a</sub>) and intraarterial blood pressures were measured during upright exercise on bicycle ergometer performed with and without concomitant arm-cranking as well as during arm-cranking alone. Arms and legs performed the same mechanical loads during all types of exercise, but arm loads were on average 40% of leg loads. It was found that  $\dot{V}O_2$  in the exercising legs (leg blood flow times femoral AVDO<sub>a</sub>) was practically the same whether the legs exercised alone or in combination with arm exercise (2.5 l O<sub>2</sub>/min. vs. 1.89 l O<sub>2</sub>/min.). Furthermore the difference between pulmonary  $\dot{V}O_2$  during combined arm- and leg exercise and  $\dot{V}O_2$  in the active legs (3.46 l O<sub>2</sub>/min. - 1.89 l O<sub>2</sub>/min. = 1.57 l O<sub>2</sub>/min.) was essentially the same as  $\dot{V}O_2$  during isolated arm-exercise (1.52 l O<sub>2</sub>/min.). This suggests unimpaired O<sub>2</sub> supply to both arms and legs during combined exercise. Nonetheless leg-blood flow was reduced by 13% when arm-exercise was superimposed. Arterial mean blood pressure was unchanged or slightly increased (113 mm Hg vs. 117 mm Hg) during combined exercise as compared to leg exercise alone. Thus addition of arm-exercise caused an increased vascular resistance in the exercising legs. The main reason for this is probably an increased sympathetic vasoconstriction in the skin in proportion to the increase in relative work load. HR increased from 174 to 192 beats per min. when arm-exercise was added to leg exercise.



## D 30

# Magnification Factor in the Foveal Region of Vervet Monkey

by C. GULD, *Institute of Neurophysiology University of Copenhagen, Denmark*

quadrant of the retina projects to striate area on the occipital lobe, the projection described by the magnification factor  $M$  mm/degree i.e. the distance in mm on cortex corresponding to 1° of visual field.  $M$  falls with increasing angle of visual field, is constant along a semicircle and describes a regular projection from center of fovea to the border of visual field (Daniel and Whitteridge 1961). Since later findings (Dow and Gouras 1973) indicate that center of fovea is represented some distance from the border of striate area, we examined the projection of fovea, 0°-4° by recording summated potentials at known locations on cortex by monochromatic small-spot flashes at known locations on retina.  $M$  was calculated for all neighbouring pairs of positions and related to distance along semicircle and angle of meridian in visual field.

Between semicircles 0.4° and 0.9°  $M$  was the same ( $P > 0.5$ ), i.e. constant along semicircles also in the foveal area.  $1/M$  as function of distance  $\theta^\circ$  from center of fovea gave the regression line  $1/M: 0.065 + 0.074 \cdot \theta$  up to 4° from fovea ( $N=273$ ,  $S_{y,x} = 0.061$ ,  $ME_0 = 0.1$ ,  $r = 0.72$ ,  $P < 0.001$ ). With  $1/M = a + b\theta$  the area on cortex is  $A = (\pi/b^2) [\ln(1 + b\theta/a) + b\theta]$  mm<sup>2</sup> ( $0 < \theta^\circ$ ). For  $\theta = 0.85^\circ$   $A = 106$  mm<sup>2</sup> compared with 113 mm<sup>2</sup> calculated from corresponding coordinates on cortex.

Our model shows that retina also in the foveal area is mapped regularly on cortex. Thus center of fovea is represented at the border of striate cortex, in this monkey about 3 mm posterior to the end of lunatus sulcus and 3 mm above the border of occipital sulcus, in agreement with the extent of striate cortex on histological preparations.

From Cowey and Rolls (1974) data on the cortical magnification factor in man, the regression line  $1/M: 0.073 + 0.053\theta$  is valid up to 15° ( $N=7$ ,  $S_{y,x} = 0.018$ ,  $ME_0 = 0.0014$ ,  $r = 0.998$ ,  $P < 0.001$ ). Thus the magnification factor of monkey is similar to that of man at the center of fovea, but it falls more steeply with increasing visual angle in monkey than in man due to the smaller total visual area.

## References

- COWEY, A. and ROLLS, E. T. *Exp. Brain Res.* 1974, 21, 447-454.  
 GULD, C. M. and WHITTERIDGE, E. *J. Physiol.* 1961, 159, 203-221.  
 DOW, B. M. and GOURAS, P. *J. Neurophysiol.* 1973, 36, 80-100.

## D 31

# Depolarization of the Cell Membrane in Rat Liver after Partial Hepatectomy

By M. PALONCHIO and M. FOLKE, *Institute of Medical Physiology Dept. A, University of Copenhagen, Juliane Mariesvej 28, DK 2100 Copenhagen, Denmark*

Numerous reports in the literature indicate that rat liver has a remarkable capacity for regeneration or compensatory hyperplasia. After a two thirds hepatectomy increased DNA

synthesis, preparatory to cell division can be detected at 12 h, mitosis at 24 hours, and by 48 h the weight of the liver remnant has doubled (e.g. Bucher *et al* 1969).

In the present study intracellular potentials were recorded *in vivo* from rat livers before and at various times after two thirds hepatectomy in order to see, whether changed membrane properties in the rapidly proliferating new cells would be reflected in such potentials. The recordings were made on fed rats weighing 195–250 g.

The potentials were in the normal preoperative range (40–44 mV) until 2–3 h after the resection, at which time an increase to 49–55 mV was seen. Apart from a transient reduction at 5–7 h this hyperpolarization was maintained for approximately 24 h. During the rest of the regenerative phase the membrane potentials were normal.

Thus, the time course of hyperpolarization does not suggest a relationship of membrane potentials to cell proliferation but rather to earlier events in the liver remnant.

The hepatic glycogen content is known to fall sharply after a two thirds hepatectomy reaching very low levels at 3–4 h (Slimek *et al* 1968). The demand for blood glucose after this time must be met by gluconeogenesis. It has recently been shown that hyperpolarization occurs in isolated perfused rat livers upon stimulation of gluconeogenesis with glucagon (Friedmann and Dambach 1973) and with various gluconeogenic substrates (Dambach and Friedmann 1974).

These reports together with our own observations raise the possibility that hyperpolarization after partial hepatectomy is associated with intensified gluconeogenesis during the early adaptation of the liver remnant to unchanged demands from the rest of the organism. Support for this hypothesis was obtained from additional experiments on rats fasted for 48 hours. In such rats hyperpolarization occurred as early as 10–20 minutes after the resection.

### References

- BUCHER, N. L. R., T. R. SCHROCK and F. L. MOULTEN, *Hepatic Med. J.* 1969 123 250–257.  
 DAMBACH, G. and FRIEDMANN, *Biochim. Biophys. Acta* 1974 367 366–370.  
 FRIEDMANN, N. and G. DAMBACH, *Biochim. Biophys. Acta* 1973 307 399–403.  
 SIMEK, J. F., F. RUBIN and I. LIEBERMAN, *Biochem. Biophys. Res. Comm.* 1968 30 571–575.

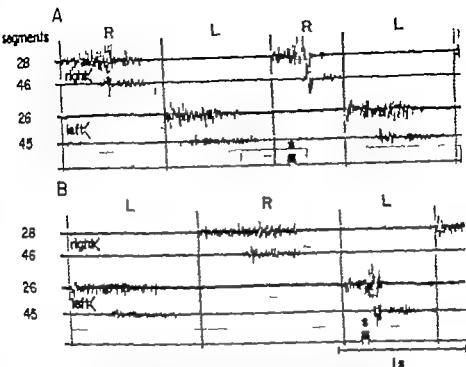
### D 32

#### Phase Dependent Reflex Reversal during Swimming in the Spinal Dogfish

By S. GRILLNER, S. ROSSIGNOL and P. WALLÉN, *Department of Physiology, University of Göteborg, Sweden*

Dogfishes can perform continuous swimming movements of the body even after a transection of the spinal cord (Gray and Sand 1946). This study is concerned with the response to a short lasting stimulation of the tail fin applied in different phases of the swimming movement.

Twelve dogfishes (*Squalus acanthias*) spinalized 4 to 8 segments below the foramen magnum were placed in a tank and fixed by the snout (see Grillner 1974). Bipolar EMG recordings were obtained from pairs of copper wires (100  $\mu$ m) implanted in the red lateral



**Fig. 1. Phase dependence of reflex response: tail fin stimulation.** The EMG from muscle segments 28 and 46 on the right and 26 and 45 on the left were recorded on Mingograph ink paper. These levels correspond roughly to the first and second dorsal fins. Note the delay of activation from rostral to caudal segments and the strict alternation between the two sides. The dotted lines divide the right (R) and left (L) sides of recording. Both sets of represents a burst of 60 ms (18 pulses of 1 ms) at 2.5 mA. Note that the stimulating electrodes were placed simultaneously on the right and the left side at the "root" of the tail fin and that the stimulation on the copper area has been removed for 3 sec on each electrode. The tail fin and the stimulation on the copper area has been removed for 3 sec on each electrode. The scale calibration is given below B.

**A.** The stimulation occurs 110 ms after the onset of the EMG in the 2nd right half cycle. A large upstroke response follows at both segments on the right side only. The duration of the EMG burst is shorter and so the corresponding half cycle. The following left half cycle is identical to the preceding one.

**B.** The same stimulation given a few seconds after the previous one but occurring 180 ms after the onset of EMG in the 2nd left half cycle. The pattern of response is the reversed image of that in A.

muscles. One such wire was inserted on each side of the root of the tail fin for stimulation. The stimulus could be delivered in any phase of the cycle through a variable delay unit triggered by the EMG activity in one channel. The fishes were filmed with a 16 mm camera synchronized with the EMG.

Fig. 1A shows the rhythmic alternating EMG activity on the two sides of the body. A short train of stimuli applied during the EMG activity on the right side gave a strong increase of the activity on this side but no contralateral effects. With an identical stimulation during EMG activity on the left side the pattern was reversed (Fig. 1B). Note that in both cases, the EMG bursts are shorter than the preceding locomotor bursts but of much higher amplitude resulting in a short but powerful stroke. Thus the same stimulus activates one side or the other in synergy with the phase of locomotion.

These results differ from those obtained with unilateral stimulation (Lissmann 1946), which only induces a "withdrawal" to the contralateral side. With the present stimulus the reflex effect depends entirely on the swimming phase. Similar observations have been made in chronic spinal cats during walking (Forsberg *et al* 1975). Such reflex reversals could be explained by activation of alternate spinal pathways, physically opened and closed by the central generator(s) for locomotion (see Grillner 1975).

### References

- FORSBERG, H. S. GRILLNER and S. ROSSIGNOL, *Brain Res.* 1975 **83** 103-107  
 GRAY J and A. SAND, *J. exp. Biol.* 1936, **13** 200-209  
 GRILLNER, S. *Exp. Brain Res.* 1974 **20** 439-470.  
 GRILLNER, S., *Physiol. Rev.* 1975 (in press).  
 LISSMANN, H. W. *J. exp. Biol.* 1946, **23** 143-161

### D 33

#### Deterioration of Drug Biotransformation in Rat Liver by a Fungicide Dichlone

By H. VAINIO and M. G. PARKKI, *Department of Physiology University of Turku, 205 20 Turku 52 Finland*

The microsomal biotransformation system metabolizes a wide variety of both exogenous and endogenous substrates to form more polar excretable end products. This occurs often in two subsequent phases, oxidation followed by conjugation, in mammals generally with glucuronic acid. The enzymes catalyzing the two enzymic steps are tightly bound to the microsomal cell fraction. Rather little is known about the topochemistry and possible linkage of the two enzyme machineries in the membrane structure. To gain further information of the above facts, the fungicide dichlone (2,3-dichloro-1,4-naphthoquinone) was administered intraperitoneally to rats at dose levels of 2, 5 and 10 mg/kg b.wt. This fungicide has been suggested to be bound to the sulphhydryl groups of the membranes, and thereby interfere with the function of *e.g.* erythrocytes (Silkka *et al* 1974).

The 10 mg/kg b.wt. dose of dichlone was already observed to be very lethal to rats, only one from eight rats did survive 24 h after the dosage. The administration of dichlone to rats caused a profound decline in the activities of the overall drug hydroxylation reactions, both hydroxylation of 3,4-benzpyrene and O-demethylation of p-nitroanisole being decreased to about 50 per cent of the control with a dose of 5 mg/kg b.wt. With this dose level NADPH cytochrome c reductase activity was decreased by 40 per cent and the content of cytochrome P-450 in liver microsomes was declined to about 50 per cent. Also the microsomal enzyme acting on epoxides, epoxide hydrolase, was inhibited similarly to the monooxygenase system.

UDP-glucucosyltransferase, the enzyme responsible for the conjugation of the (pre-formed) hydroxylated products has been suggested to have a deep location in the microsomal membrane, perhaps in its inner compartment (Vainio 1973). Its activity was not detectably altered by dichlone administration to rats, not even in microsomes treated *in vitro* with digitonin and trypsin to detect also the latent transferase activity

The above results indicate that the fungicide dichlone is rather toxic for rats, the lethal being about 10 mg/kg b.wt. Of the microsomal biotransformation reactions, mono-oxygenase and epoxide hydrase were readily affected by dichlone administration whereas glucosyltransferase was more resistant towards dichlone treatment. Thus the present data support our earlier assumptions on the more protected microenvironment of glucosyltransferase in the microsomal membrane than the mono-oxygenase system (1973).

### References

- A. R. C. SCHWARTZ, E. H. SAKUMA, J. and ZWISLOCKI, G., *Abstract Book, The Third International symposium of Pediatric Chemistry* Helsinki 1974 228.  
 de, H. On the Topology and Substrates of Drug-Metabolizing Enzymes in Hepatic Endoplasmic Reticulum. *Thieme, Tübingen*, 1973. Pp. 3-46.

### D 34

#### Algebraic Biotransformation of Xenobiotics in the Gunn Rat

By J. MARNETT, H. VANDER and M. G. PARKER. *Department of Physiology University of Turku, 20520 Turku 52, Finland*

The Gunn strain of the conventional Wistar rat is known to exhibit congenital non-soluble hyperbilirubinemia due to an almost complete lack of the activity of the enzyme UDPglucosyltransferase towards bilirubin (Carbone and Grodsky 1957). On the other hand, Gunn rat is able to glucuronidate some exogenous compounds, e.g. p-nitrophenol virtually normally (Dutton, 1971).

In the present study we investigated glucuronyl, glucosyl, glutathione and glycine conjugations and epoxide hydration in livers of Wistar and Gunn rats to clarify the possible operational mechanisms of the drug biotransformation and to further study the nature of the glucuronidation defect in the Gunn rat.

The activities of bilirubin UDPglucosyl- and UDPglucosyltransferase were in hepatic microsomes of homozygous Gunn rats less than 10% of those in Wistar rats. In heterozygous Gunn rats the activities were 60-70%, smaller than in Wistar rats. Treatment of microsomes *in vitro* with digitonin, trypsin or phospholipase C enhanced the measurable activities of UDPglucosyl- and UDPglucosyltransferase with equal relative magnitude in all three groups of animals. When 4-methylumbelliferone was used as the aglycone substrate of UDPglucosyltransferase the activity of the enzyme was in non-treated microsomes of homozygous and heterozygous Gunn rats over 60% of that in Wistar rats. Preincubation of the microsomes activated also the enzyme of the Gunn rats but did not bring it to the level obtained in pretreated microsomes of Wistar rats.

The activities of hepatic microsomal epoxide hydrase and the supernatant enzyme glutathione S-epoxide transferase (both determined with styrene oxide as substrate) did not differ significantly in Gunn rats from the values of Wistar rats. The activity of glycine (4-acyl)transferase of isolated mitochondria was, however, in Gunn homozygotes significantly (about 85%) higher than in Wistar rats. Also in heterozygotes the activity appeared to be slightly higher (about 33%) than that in Wistar rats.

The reciprocal velocity (Lineweaver-Burk) plots of hepatic UDPglucuronosyltransferase with varied UDPglucuronic concentrations bent downwards at low UDPglucuronic acid concentrations similarly in microsomes of Wistar and homozygous and heterozygous Gunn rats when determined with  $^4\text{C}$  labeled p-nitrophenol as substrate. Thus the kinetics of UDPglucuronosyltransferase appeared to be similar in normal and jaundiced rats in spite of the lower activity of the latter ones.

Our results indicate that the hyperbilirubinemic Gunn rats are as deficient in UDPglucosyltransferase as in UDPglucuronosyltransferase activity towards bilirubin. Other drug detoxifying enzymes studied were, however at normal or even elevated level in Gunn rats. The kinetic and activation studies supported the concept of normal microenvironment of microsomal UDPglucuronosyltransferase in the Gunn rat.

### References

- CARBONE, J. V. and G. M. GRODSKY *Proc Soc exp. Biol (N.Y.)* 1957 94 461-463.  
DUTTON, G. J. *Handbook of Experimental Pharmacology* 1971 XXVIII. Springer Berlin-Heidelberg-New York, pp 378-400.

### D 35

#### Effect of Beta-receptor Blockade on Heart Rate, Hepatic Blood Flow and Circulating Noradrenaline during Exercise in Man

By N. J. CHRISTENSEN, J. TRAP-JENSEN, J. P. CLAUSEN, I. NOER, A. R. KROGSGAARD and O. ANDRÉAS LARSEN *Second Clinic of Internal Medicine Kommunehospitalet Århus, DK-8000 and Departments of Internal Medicine M and Clinical Physiology Frederiksberg Hospital DK-2000 Copenhagen, Denmark.*

Beta-receptor blocking agents cause a reduction in heart rate, in cardiac output and in arterial blood pressure both at rest and during exercise. The impaired cardiac response to the sympathetic stimulation during exercise could be anticipated to cause a compensatory increased peripheral vasoconstriction.

The present investigation was undertaken to study the effect of long-term beta-receptor blockade on heart rate (HR), on peripheral vasoconstriction as judged by changes in the splanchnic-hepatic blood flow (SBF) and on arterial plasma noradrenaline concentration (NA). HR, SBF and NA were measured at rest supine and during 20 min of upright submaximal exercise on a bicycle ergometer in six male subjects with mild essential hypertension (mean age 35 years) before and after 4 to 8 months daily administration of 160 to 320 mg propranolol perorally. The subjects exercised at the same absolute work loads during both studies. Catheters were placed in the main right hepatic vein and in the common iliac artery. ECG was continuously recorded, SBF was measured using constant infusion of indocyanine-green. The NA concentration was determined using a precise and sensitive double isotope derivative technique.

During exercise the reduction in SBF was accompanied by an increase in NA from rest to exercise ( $\Delta \text{NA}$ ) and SBF expressed as per cent of the value at rest ( $\text{SBF}\%$ ) was closely correlated to  $\Delta \text{NA}$  ( $p < 0.01$ ). The increase in HR over the value at rest ( $\Delta \text{HR}$ ) was also

only correlated to SBF% (Clausen and Trap-Jensen, 1974 Rowell, 1974) as well as to ... (Christensen and Brandeborg, 1973).

ing beta-receptor blockade the relation between SBF% and  $\Delta$  NA remained unchanged although SBF in mean was 33 per cent lower and  $\Delta$  NA was increased by a factor of 2 at the same oxygen uptake. In contrast the relation between  $\Delta$  HR and  $\Delta$  NA showed no displacement to the left by about 50 beats per min.

present observations showed that during exercise different parameters (HR, SBF (A) reflecting the degree of activation of the sympathetic nervous system changes proportion to each other. They showed in addition that the suppression of the cardiac output during exercise caused by beta-receptor blockade leads to a compensatory alpha-receptor mediated constriction of resistance vessels in non-working tissues with a concomitant proportional increase in circulating NA.

### References

- FRID, N. I. and BRANDENBERG, O., *Europ. J. clin. Invest.* 1973, 3, 299-306.  
 DE J. P. and TRAP-JENSEN, J. *J. appl. Physiol.* 1974, 37, 716-719  
 L. L. B. *Physiol. Rev.* 1974, 54, 75-139

### D 36

**Changes at Supramaximal Ventilations during Rebreathing and Steady State Exercise**  
 by JAKOB MERTZ, POU-ERIK PAULEV and S. TORBEN STENYANG PEDERSEN, *Institute of Medical Physiology B, University of Copenhagen, Denmark*

There is a linear relation between pulmonary ventilation ( $\dot{V}_E$ ) and tidal volume ( $V_T$ ) during steady state  $CO_2$  breathing at rest and during moderate exercise, when breathing at a  $V_T$  equal to approximately half the vital capacity (Hey *et al.* 1966). With further increases in  $\dot{V}_E$  (up to 80 L (BTPS)/min.) Hey *et al.* found no further increase in  $V_T$ . Similar results were obtained in initially eupnoeic cats (von Euler, Herrero and von Euler 1966). During rebreathing in man, the frequency ( $f$ ) was found rather constant at ventilation up to 30 L/min, range 1, and with further increments in  $\dot{V}_E$  a concomitant, almost 2 fold rise in  $f$  was observed, range 2 (Clark and von Euler 1972).

Respiration was measured breath-by-breath with a system described earlier (Paulev 1971). End-tidal gas tensions were obtained with Beckman instruments.

Our subjects were examined during rebreathing and cycling at different work rates. In these experiments a range 1 was not observed, but at supramaximal ventilation, a fall in  $f$  and a rise in  $V_T$  was obtained (Fig. 1). At the highest steady state work rate (1800 W/min)  $\dot{V}_E$  reached ventilations up to 200 L (BTPS/min) (e.g. actually the average, alveolar volume-rates per breath) at end-tidal  $P_{O_2}$  ( $P_{aO_2}$ ) values just above 100 mm Hg. These ventilations exceed the maximal voluntary ventilation measured in 15 seconds ( $\dot{V}_{E_{max}}$ ).

At the supramaximal ventilations, the  $V_T$  was almost constant at approximately 170 L (BTPS), while the  $f$  decreased 1-1.5 L (BTPS) with a corresponding increase in  $f$  to

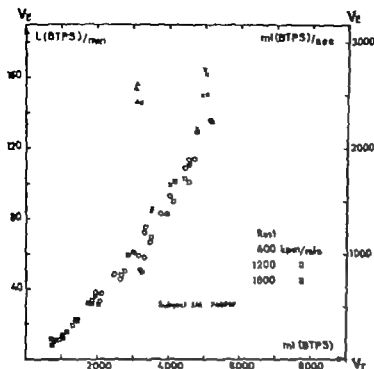


Fig. 1 The expiratory volume  $V_E$  is shown in relation to the expiratory tidal volume,  $V_T$ , for each breath: one subject.

45–50 breaths/min in all 4 subjects. The explanation of the present phenomenon (i.e. the fall in  $V_E$  and the rise in  $f$  at supra maximal ventilations) may include one of the following possibilities or their combination

First, at high levels of inspiratory flow an "intercostal muscle-phrenic" reflex derived from the intercostal muscle spindles in response to chest wall distortion cause an inhibition of phrenic activity in cats and dogs (Remmers 1970)

Secondly we observed a rise in rectal temperature of approximately 0.5 C from rest to work at 1800 kpm/min. Part of the "shift to the left" in the  $V_E$  vs.  $V_T$  plot, which was observed at the two highest work rates (Fig. 1) may be explained by a temperature effect (Vejby-Christensen and Strange Petersen 1973). Hey *et al* (1966) found, that raised temperature is the only respiratory stimulus, which affects the  $V_E$ - $V_T$  relation. Since the  $V_T$  was falling more and more ( $\Delta$  in Fig. 1) with increasing  $P_{a\text{CO}_2}$  during the last 45–60 seconds of the exercise—rebreathing combination a  $P_{\text{O}_2}$  stimulus must also be involved.

### References

- CLARK, F. J. and EULER, C. VON *J. Physiol.* 1972, 222, 67–79.  
 EULER, C. VON, HERRERO, F. and WEXLER, I. *Res. Ph. sci.* 1970, 10, 93–108.  
 HEY, E. N., LLOYD, B. B., CUNNINGHAM, D. J. C., JONES, M. G. M. and BOLTON, D. P. G. *Res. Ph. sci.* 1966, 1, 193–203.  
 PAULSEN, P. E. *J. appl. Ph. sci.* 1971, 30, 165–172.  
 REMMERS, J. E. *Res. Ph. sci.* 1970, 10, 358–383.  
 VEJBY-CHRISTENSEN, H. and STRANGE PETERSEN, E. *Res. Ph. sci.* 1973, 19, 322–332.



## D 37

**pO<sub>2</sub> Sensitivity of Spontaneous and Induced Contractions in Venous Smooth Muscle**  
 by PER HELLESTRAND, *Department of Physiology and Biophysics, University of Lund, Sweden*

contractile response of arterial smooth muscle to adrenergic stimulation depends on (Detar and Bohr 1968, Pittman and Duling 1973). It is of interest, however to investigate also the effect of low pO<sub>2</sub> on vessels showing myogenic tone, since variation of such is important for regulating flow in the microcirculation (e.g. Mellander and Johansson 1968). Flow resistance of an isolated arterial segment has been shown to vary with pO<sub>2</sub> (Walter and Guyton 1964). For an understanding of the cellular mechanisms of this dependence however direct study of the vascular smooth muscle is needed. In the present work the influence of pO<sub>2</sub> on spontaneous and induced contractions of the isolated portal vein has been determined.

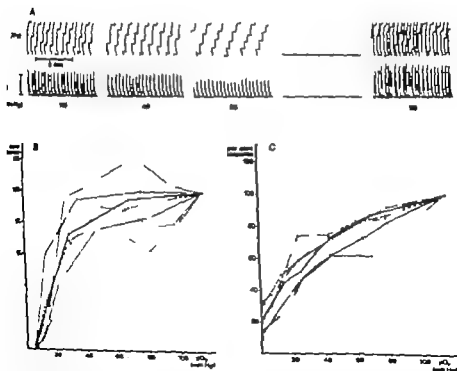


Fig. 1. (A) Patterns of spontaneous activity appearing in portal vein at 5 different levels of pO<sub>2</sub> applied (from left to right: Lower record: tension; Upper record: time-integral of tension (arbitrary units, note interval)). Note increased activity as oxygen is readmitted after hypoxia. (B) Integrated spontaneous activity at successively lower pO<sub>2</sub> 7 preparations. Each point represents integral over 15 min as a percentage of control response for same muscle at 115 mm Hg. (C) Contracture tension on 2 mM K<sup>+</sup> depolarization as function of pO<sub>2</sub> 6 experiments. Successively lower pO<sub>2</sub> applied. 15-30 min between stimulations. Control response for each muscle set at 100 mm Hg.

Longitudinally opened veins (wall thickness 100–150  $\mu$ ) were mounted in a bath. The patterns of spontaneous activity appearing as  $pO_2$  was lowered in steps from 115 mm Hg to 1 mm Hg are exemplified in Fig. 1A. At reduced  $pO_2$  both duration and amplitude of each phasic contraction were diminished. After the hypoxic period there was a transient increase of activity lasting about 20 min. In Fig. 1B the time integral of tension is plotted against  $pO_2$ . A steep dependence is noted in the range below 50 mm Hg. The sustained tension developed on K<sup>+</sup>-depolarization is also oxygen-dependent, as shown in Fig. 1C. However at 1 mm Hg the muscles were still capable of developing  $23.2 \pm 3.3\%$  (mean  $\pm$  S.E.,  $n=6$ ) of control tension. Maximal stimulation with noradrenaline at 1 mm Hg gave an integrated tension of  $26.9 \pm 1.7\%$  of control response.

This study has shown a marked oxygen dependence of the myogenic activity of a vascular smooth muscle preparation in ranges of  $pO_2$  occurring physiologically. The effect does not seem to be entirely due to depletion of energy supplies needed for contraction, since hypoxia appears to cause also an inhibition of membrane events triggering the spontaneous contractions.

### References

- CARRER, O. JR., J. R. WALKER and A. C. GUYTON, *Amer J Physiol* 1964, 206, 951–954.  
 DETAR, R. and D. F. BOHR, *Amer J Physiol* 1968, 214, 41–44.  
 MELLANDER, S. and B. JONLIDSON, *Pharmacol. Rev* 1968, 20, 117–196.  
 FITTMAN, R. N. and B. R. DULING, *Microvasc. Res* 1973, 6, 202–11.

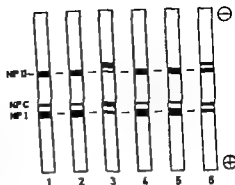
### D 38

#### Heat Inactivation of Bovine Neurophysins

By HANS VILHARDT and MICHAEL HOLGATE. *Institute of Medical Physiology C University of Copenhagen, Denmark and Department of Pharmacology University of Oxford, England*

The neurophysins are low molecular weight proteins found in the neural lobe of the pituitary gland. They are characterized by their ability to bind the hormones vasopressin and oxytocin. In the bovine neurohypophysis three such proteins have been isolated and named neurophysin I, C and II in order of decreasing electrophoretic mobility at pH 8.1 (Hollenberg and Hope 1968, Rauch *et al* 1969).

In the present study extracts of fresh bovine neural lobes were subjected to polyacrylamide gel electrophoresis. The electrophoretic mobility of the neurophysins was identical to the mobility of neurophysins purified from preparations of acetone-dried bovine posterior pituitaries (Fig. 1). If however the extract of fresh neural lobe tissue was heated (100 °C for 20 min) a change in the mobility of neurophysin I and II on the gels was found. This was not observed when solutions of purified neurophysins were treated in the same way. Since neurophysins are rich in disulfide bonds it was possible that the fresh extracts contained reducing agents and that heat treatment accelerated a reduction of the neurophysins. To test this hypothesis fresh extracts were heated in the presence of 5 mM potassium ferri cyanide. The presence of this oxidant in the extract completely inhibited the degradation of



polyacrylamide-gel electrophoresis of bovine sera. 1) neural lobe extract, 2) purified sera, 3) heat treated neural lobe extract, 4) heat treated purified neurophysins, 5) neural lobe extract treated in the presence of  $K_4Fe(CN)_6$ , 6) neurophysins heat treated in the presence of  $K_4Fe(CN)_6$ .

350 I and II When a solution of purified neurophysins was heat treated in the presence of 2 mM glutathione a change in the electrophoretic mobility could be demonstrated. It is concluded that extracts of fresh neural lobe tissue contain reducing substances and the effect of heating of the extracts could be reduction of the disulfide bonds of neurophysins or mixed disulfide formation. Finally it could be demonstrated by affinity chromatography that the heat induced changes of the neurophysins had abolished their binding to asopressin.

### References

- BRADY, M. D. and D. B. HORN. *Biochem. J.* 1968. 106. 357-364.  
BRADY, M. D., HOLLANDER, and D. B. HORN. *Biochem. J.* 1969. 113. 473-479.

### D 39

Star Transport of Proteins from Blood to Brain during Acute Hypertension  
by HANS E. BRUNDSTED and E. WESTERGAARD. Department A of Medical Physiology and Department C of Anatomy University of Copenhagen, Denmark

intravenously injected horseradish peroxidase (HRP) has been demonstrated to cross, by star transport, the walls of very few and short segments of cerebral arterioles, capillaries and venules in mice and rats (Westergaard 1974a, Nærvæd and Westergaard 1974).

During acute hypertension (HT) evoked by metaraminol bitartrate (Aramine) the blood-brain permeability to Evans Blue and to HRP is increased (Olsson and Hassmann 1970, Olsson 1974).

The purpose of this study was to see if acute HT increases the modest vesicular transport observed in cerebral vessels and to identify the routes taken by the protein tracer. The arterial blood pressure (BP) was continuously recorded from the femoral artery of 8 rats anesthetized with sodium-pentobarbitone. During the first 40 min the BP was 120 mmHg. Aramine was infused i.v. at 0.07 mg per kg per min immediately resulting in a well-defined BP of 170-190 mmHg. 10 min later 100 mg of HRP (Sigma, type II) was slowly infused and circulated for 15 min followed by cardiac perfusion with aldehydes.

The brain was removed, cut into slices and incubated. Some of the tissue sections were cleared. Other samples were processed for electron microscopy.

The cleared sections showed a very marked increase of reaction product as compared with controls not receiving Aramine, both in the walls of many larger vessels, in the neuropil and in the subarachnoid space.

Electron microscopic examination showed reaction product in the walls of many arterioles and venules, in the basement membranes under their endothelium and in surrounding smooth muscle cells, in the perivascular spaces and in the intercellular spaces of the neuropil nearby.

Higher magnification showed that HRP did not pass the first luminal tight junction. The intercellular spaces were filled retrogradely with HRP from the subendothelial basement membrane up to the first abluminal tight junction. The cytoplasm of endothelial cells contained several vesicles filled with HRP. No endothelial cell damage was demonstrated.

In conclusion, acute HT induced by Aramine strongly increased the normally occurring vesicular transport of HRP through the walls of cerebral vessels while the tight junctions effectively prevented any escape of HRP between endothelial cells. However, beside any effect of HT upon the vesicular transport it cannot be ruled out that Aramine itself might induce vesicular transport since it has recently been shown that serotonin and norepinephrine with molecular structures resembling that of Aramine both induce vesicular transport (Westergaard 1974 b).

### References

- JOHANSSON, B. *Blood-Brain Barrier Dysfunction in Acute Arterial Hypertension*. Gotab, Kungälv, Sweden, 1974.
- NORTVED, J. and E. WESTERGAARD. *Acta Rec* 1974, 178, 428.
- OLSSON, Y. and K. A. HOMMANN. *Acta Neurologica* (Berl.) 1970, 16, 117.
- WESTERGAARD, E., in *Pathology of Cerebral Microcirculation*, (ed. J. Cervós-Narvá), 1974 a, p. 18.
- WESTERGAARD, E., *Ultrastuct. Res.* 1974 b. In press.

### D 41

#### An Electrode for Micropuncture Measurements of $P_{CO_2}$

By BERTIL KARLMARK and MORGAN SOMTELL. *Department of Physiology and Medical Biophysics, Biomedical Center, University of Uppsala, Sweden*

When studying metabolic and transport processes in living tissue it is of great importance to take into account the tissue handling of acid and bases. Regarding the bicarbonate system, very little is known about its regulation at the cellular level. When carbon dioxide is produced, it will give rise to bicarbonate and hydrogen ions in relation to other intracellular as well as extracellular buffers. The transport of carbon dioxide across a barrier thus also includes the transport of bicarbonate and hydrogen ions. Micropuncture investigations of transport processes for the components in the carbon dioxide system at a cellular level has, for technical reasons, been undertaken in an indirect manner requiring knowledge of the actual in vivo pH. In such experiments nano-liter samples are taken for equilibration

shows carbon dioxide tensions and the actual values of bicarbonate and  $P_{CO_2}$  can be obtained from the buffer curve obtained. The evaluation of the bicarbonate transport system are furthermore mainly based upon the hypothesis, that the carbon dioxide is in the extracellular space and in the cells of the proximal tubule of the kidney equal to that in the arterial blood. However an increasing amount of information questioning this identity. These findings might mean that the bicarbonate buffer system is a chemical equilibrium and therefore the variables have to be individually measured. An electrode for the direct measurements of  $P_{CO_2}$  in tissue was constructed and was used for the use in proximal tubular micropuncture experiments on the rat kidney. It is of an  $Sb/Sb_2O_3-Ag/AgCl$  electrode system for measurements of the pH in a artificial bicarbonate solution. This solution together with the electrodes are enclosed in the tip of the cannula with a tip diameter of about 6  $\mu m$ . The very tip is closed with a membrane which is diffusible for carbon dioxide. The pH sensitive surface of the antimony electrode is placed in the bicarbonate solution just near the membrane. The response time of the electrode is less than 3 min and the mean sensitivity is more than 90% of the expected value.

## D 42

### Measurement of the Norepinephrine Content in Sympathetic Nerve Terminal

By H. LAGERCRANTZ, G. FRIED and T. HÖKfelt *Departments of Physiology and Histology Karolinska Institute, Stockholm, Sweden*

Norepinephrine (NA) content in each sympathetic nerve terminal vesicle is of interest for the discussion of quantal release from nerve terminals. However direct determinations of the NA content in nerve terminal vesicles have not been done since pure preparations of these particles are not yet available. The aim of the present study is to describe possible experimental methods to estimate the NA content in both large dense-cored and small dense-cored vesicles occurring in the nerve varicosities.

Large nerve vesicles were isolated (80-90% purity) from bovine splenic nerve axons by sequential and density gradient centrifugation (see Lagercrantz, Kirksay and Klein 1974). To estimate the NA content in the terminal vesicles the ratio between NA and dopamine  $\beta$ -hydroxylase was determined in different segments of the axons and in crude large vesicle preparations from the spleen. Furthermore the NA loss during the post-mortem delay had to be considered e.g. the time interval between the animals were killed until the nerves could be chilled on ice. Therefore the nerves and spleens were taken at different post-mortem times and the NA/protein ratios in the different preparations were plotted to the moment the animals were killed.

Estimation of the NA content in the small nerve vesicles was carried out on preparations of terminal ducts from normal and castrated male rats. By castration of the rats the muscle atrophied after about 3 weeks while the nerve varicosities were still intact. The small nerve vesicles were isolated by differential and density gradient centrifugation essentially as described by Bisby and Fillenz (1971).



Fig. 1 NA storage particles isolated from seminal ducts of castrated rats by differential centrifugation (10 000 g for 30 min) followed by allowing the obtained supernatant directly on density gradients (0.25–0.8 M sucrose) which then were ultracentrifuged. The fraction with the highest NA and dopamine  $\beta$ -hydroxylase activity (about 0.45 M) as diluted to isotonicity and mixed with glutaraldehyde and then centrifuged at a high speed. The pellet was then postfixed and embedded.

The NA/protein ratio was estimated to be 65–85  $\mu\text{g}/\text{mg}$  protein in the large nerve vesicles in the bovine spleen corresponding to  $2\text{--}4 \cdot 10^{-4}$  ng NA/vesicle. The NA/protein ratio in the small nerve vesicle preparations of the rat vas deferens from normal rats was found to be  $892 \pm 50$  (S.E.,  $n = 7$ ) ng/mg protein and  $2796 \pm 705$  (S.E.,  $n = 3$ ) ng/mg from the castrated rats. The highest ratio was 4349 ng/mg.

A micrograph (Fig. 1) of the vesicle preparation from vas deferens of the castrated rats shows quite a high occurrence of small dense-cored vesicles indicating considerable lower NA content in these vesicles than earlier estimated by Dahlström, Häggendal and Hökfelt (1966). They estimated the NA content per vesicle to be  $2\text{--}4 \cdot 10^{-4}$  pg on the basis of analyses of the number of varicosities and NA content per unit tissue of rat vas deferens.

### References

- BERRY M. A. and M. FILLENZ, *J. Physiol.* 1971 215 163–179.  
 DAHLSTRÖM, A., J. H. HÄGGENDAL and T. HÖKFELT, *Acta Physiol. Scand.* 1966 67 289–294.  
 LAGERCRANTZ, H., B. F. ABRUMBY and R. L. KLEIN, *J. Neurochem.* 1974 23 769–773.

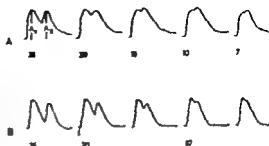
### D 42

#### Summation of Receptor Potentials in the Retina of the Fly *Calliphora* Studied by Very Short Double Flashes

By M. JÄRVILEHTO and J. MORING, *Department of Physiology, University of Oulu, Finland*

Some studies on receptor potential summation have shown that the second potential response amplitude can be either increased or depressed (mechanoreceptors, Gray and Sato 1953; stretch receptors, Ottoson, McReynolds and Shepherd 1969; light receptors, Duncan and Croghan 1973). These relations were studied in the visual receptor cells of the fly

1. Two potential series related from two different receptor cells ( $R_1$  and  $R_2$ ). In (A) the second response amplitude ( $A_2$ ) is increased and in (B) depressed by the 25% stimulus. Stimulus: 10  $\mu$ s light at 15% interstimulus interval.



Dark-adapted retinal receptors were stimulated by very short (10  $\mu$ s) double light in a stroboscope (Soc. AN-NA, Milano), programmed by a pulse generator. The potential responses were recorded intracellularly from reticular cells by capillary microelectrodes. The cells were identified by a histological method staining with Procion yellow and localization of the recording site by freeze fracture and Zentgraf (1973).

The present results are based on 12 receptor cells. The summation of the two responses is not consistent. In six cases the second response amplitude is depressed, it is increased (Fig. 1). If the interstimulus interval (ISI) is 100 ms or more, the ratio of the amplitudes of the two responses ( $A_2/A_1$ ) is about 1.0. By very short ISIs (7–15 ms) in some receptors more than 1.0 and in some less than 1.0.

Related to the high flicker fusion frequencies obtained in optomotor studies, it is to distinguish the response to the second stimulus from the potential complex.

No correlation could be found between polarization sensitivity and  $A_2/A_1$  ratio. The differences between the ratios in various cells could be explained on the basis of similar adaptation mechanisms in the receptors.

## References

- ANON, G. and CHODURA, P. C. *Structure and Physiology of Visual Pigments* (ed. Springer, New York, 1973).
- DAI, J. A. B. and SATO, M. *J. Physiol.* 1953, 102, 610–634.
- LEWIS, H. and ZETTL, R. F. *Z. Zellforsch.* 1973, 136, 291–306.
- STROM, D., MCRATON, J. E. and SIKKES, G. M. *J. Neurophysiol.* 1969, 32, 24–34.

**Regulation of Cell Growth and Prolactin Production in Cultured Rat Pits**  
By H. TERNSTADT, E. HAUG<sup>2</sup> and K. M. GAUTVIK. *Institute of Chemistry, University of Oslo, Norway; Institute of Physiology, University of Oslo, Norway; and Hormone and Isotope Laboratory, Akershus Hospital, Oslo, Norway.*  
A deoxyinosine-activated 3' and 5'-nucleotidase which is present in most tissues, has been suggested to be involved in the control of DNA/RNA synth

entiated cellular functions is insufficiently understood. Monolayer cultures of differentiated rat pituitary cells (GH<sub>3</sub>) synthesize and secrete prolactin (PRL) into the culture medium (Tashjian and Hoyt 1972).

In spontaneously growing cells and in oestradiol treated cultures we have measured the intra and extracellular concentrations of PRL as well as the nucleotidase activity. The different phases of cell growth were monitored by measurements of total cell protein and incorporation of <sup>3</sup>H thymidine into DNA. The GH<sub>3</sub> cells grow exponentially for about 8 days, after which they reach a plateau phase where cell growth and <sup>3</sup>H-thymidine incorporation into DNA are greatly reduced. During exponential growth the nucleotidase activity was unchanged, in contrast to the spontaneous rate of extracellular PRL accumulation which increased about 3 times. At the early plateau phase there was a rapid decline in PRL production rate to near initial values. During this growth phase the nucleotidase activity was doubled and the rate of DNA synthesis reduced by 50%. When the cells were cultured in the presence of 17  $\beta$ -oestradiol (10<sup>-6</sup> M) the rate of PRL production increased more than 100% with no change in the nucleotidase activity. The incorporation of <sup>3</sup>H thymidine into DNA was reduced by the oestradiol treatment.

These experiments show that the hormone production is most efficient when the cell is about to diminish the synthesis of macromolecules important for cell growth and division. Oestradiol treatment did not alter this pattern of basal hormone production, but it was able to stimulate PRL production at all different growth phases.

## References

- FRIETSON P. *Eur J Biochem* 1972, 27, 38-44.  
TASHJIAN A H Jr and R. F. HOYT Jr. *Proceeding of the Symposium on Molecular Genetics and Developmental Biology* (ed. M. Sussman). Prentice-Hall, New Jersey 1972. Pp. 353-387.

## D 45

### Persistent Effects of Large Doses of Electrolytes in the Food of Lactating Rats on the Kidney Function of Their Offspring

By A. FREDRIKSSON and U. SÖDERBERG. *Neurophysiological Laboratory, Ulleråker Hospital, Uppsala, Sweden*

The significance of birth in developmental endocrinology has received increasing attention since the discovery that hormonal conditions at birth will determine maturation of endocrine regulatory systems with serious permanent consequences (e.g. Harris and Levine 1965). Physiological changes at birth (Dawes 1968) profoundly influence the endocrines of the newborn due to cessation of maternofetal correlations and placental action. In addition endocrinal consequences of new nutritional conditions exist (Jost and Picon 1970). This investigation was prompted by the finding of a reduced rate of elimination of radioiodine from offspring of rats treated with lithium during pregnancy and lactation (Söderberg 1973). Even if renal handling of electrolytes is impaired in hypothyroid rats (Michael *et al.* 1972) and lithium interferes with thyroid activity the observed extrathyroidal effects might have been induced outside the pituitary-thyroid axis. Therefore, lithium and sodium chloride



nched diets were tested against a standard diet in pregnant and lactating rats. The offspring (n 207) were allowed to reach adult age, then studied for fate and distribution of iodine.

In contrast to lithium action on thyroid parameters, the effects on extrathyroidal iodine were found to depend on salt, similarly after equimolar amounts of lithium and sodium iodide. Further the effects were much heavier in animals treated only during lactation, plasma levels of radiolodine (4 h after a single dose) being 35 and 37% higher than in male and female controls respectively. There was also a significant 8% reduction in kidney weight. Serum levels paralleled iodine levels in muscle, liver, kidney and testicular tissues as well as thyroid glands of the lithium rats. (Fagerberg, Fredriksson and Söderberg, to be published.)

The finding that salt load of lactating rats influenced the off-spring with permanent impairment of renal function may be either an effect of transient endocrine changes in the mothers or a direct action on the newborns. Rats are very sensitive to salt excess, but it is an open question whether or not other hormones than aldosterone are also involved (Levinson 1974). Assuming that some transient changes in hormones responsible for electrolyte regulation is induced during a critical period of the development of the offspring, changes in maturation of regulatory systems might take place in analogy with changes in pituitary-renal and pituitary-thyroid system by other hormones. If dehydration, then, will cause similar effects as salt load, the present results have to be considered also in interpreting experiments on other disturbances in suckling behaviour and maternal water intake.

### References

- DALE, G. S. *Fetus and Neonatal physiology: A comparative study of the changes at birth*. Year Book Publ., Chicago, Ill. 1968.  
 HARRIS, G. W. and S. LEVINE, *J. Physiol.* 1965 121 379-400.  
 JEE, A. and L. PICO, *Advanc. Metabol. Disord.* 1970, 4 123-184.  
 MCCOY, U. F., R. L. BARNHART, R. CHAVEZ, C. A. VAAMONDE and S. PAPPAS, *J. Clin. Invest.* 1972, 51 1405-1412.  
 LEVINE, M. G. *Advanc. Metabol. Disord.* 1974, 7 37-71.  
 LUTERBACH, U. *Word ps. Amer. Table* 1972, 27 414-419.

### D 46

#### Energy Metabolism in Different Human Skeletal Muscles during Voluntary Isometric Contraction

By G. BOLSTAD and A. ERSLAND, *Institute of Physiology University of Bergen, Norway*  
 Studies on isolated fast-twitch muscles at 2°C have suggested a higher energy turnover in m. biceps than in m. soleus at comparable tensions. (Goldspink *et al.* 1970). The present study was undertaken to clarify whether similar differences exist amongst different skeletal muscles in man.

The rate of temperature increase was measured during maximal voluntary isometric contraction (MVC) and circulatory arrest in m. soleus, m. sacrospinalis and m. biceps in 22 male students. Nylon-insulated 36-gauge copper/constantan thermo-elements were in-

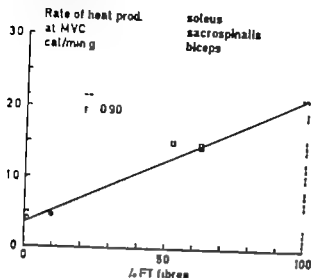


Fig. 1 Maximal rate of heat production of different muscles related to percentage of fast twitch (FT) fibres.

inserted 5-6 cm into the muscle through a hypodermic needle. Provided no heat is lost from the monitored region the local heat production in the muscle can be calculated as  $\dot{Q} = \Delta T / \Delta t \cdot q$  cal/min g, where  $\Delta T / \Delta t$  is the temperature rise per minute and  $q$  is the specific heat of muscle estimated as 0.9 cal/g.

$\dot{Q}$  rose in all experiments with increasing force of contraction. The mean rate of heat production at MVC was 0.55 cal/min g (range 0.29-0.90,  $n=11$ ) in soleus, 0.66 cal/min g (range 0.41-1.07,  $n=6$ ) in sacrospinalis and 1.48 cal/min g (range 1.30-1.71,  $n=5$ ) in biceps.

Muscle biopsies were taken from 14 muscles and fibre type distribution determined histochemically by staining for ATPase at pH 9.4.

The rate of heat production at MVC showed positive correlation to the area of fast twitch (FT) fibres given as percent of the cross-section area of the muscle (Fig. 1). The regression line is given by  $\dot{Q} = 0.018 \cdot \% FT + 0.35$ ,  $r = 0.90$ .

Linear extrapolation suggests that a muscle composed exclusively of FT fibres would have a maximal heat production of 2.15 cal/min g, whereas a muscle containing slow twitch (ST) fibres only would have a maximum of 0.35 cal/min g.

Thus, according to this estimate, the maximal energy turnover in human FT fibres is approximately six times that of ST fibres during voluntary isometric contraction.

### Reference

CHAMPAK, G. R. E. LARSON and R. E. DAVIS, *J. ergot Physiol* 1970 66 389-397

### D 47

#### Metabolism of Iodine and Iodinated Thyroid Products in Amphibia

By P. ROSINKILDE, Zoophysiological Laboratory A University of Copenhagen, 15 Universitetsparken Denmark

A survey of thyroid hormone dynamics in adult amphibia is attempted. The most complete investigation was made on the common toad *Bufo bufo*. I 131 uptake, total iodine

concentration and relative distribution of iodinated products in plasma, and biological half-life of I-125 labelled thyroxine. Further it was attempted to obtain isotopic equilibrium of all iodine compartments by injection of I 125 followed by repeated injections in an amount equal to those excreted by the toads. Similar though less complete, determinations made on the African clawed toad, *Xenopus laevis*, and the Mexican axolotl, *Ambystoma mexicanum*.

Thyroidal uptake of radioactive iodine has been measured many times in several amphibian species (See Rosenkild 1969-1974). A typical value for *B. bufo* is 18-20% of injected amount. *Xenopus* takes up much less (usually 2-4%). Axolotls in their natural occurring, larval (neotenic) form also have low iodine uptakes (1-5%). (Artificially) metamorphosed axolotls have varying levels of thyroid function from about nil with corresponding absence of thyroidal iodine uptake, up to values similar to those of other naturally metamorphosed urodeles (20-30%).

Plasma iodine concentration in plasma shows large interspecific variations. *B. bufo* usually around 0.01 µg/ml (range 0.00-0.03), comparable concentrations are found in larval and metamorphosed axolotls. Larval, 0.03, metamorphosed, 0.06 µg/ml, while *Xenopus* plasma contains 0.20 µg/ml. The difference only partly corresponds to the different iodine content of the food. *Xenopus* and larval axolotls are fed pieces of mouse (0.3 µg I/mg) while metamorphosed axolotls and *B. bufo* eat mealworms (usually less than 1 µg I/100 mg).

The distribution of iodinated products was followed in isotopic equilibration experiments. In the thyroid, changes were only observed during the first 3-5 weeks, after which the thyroid contained 25% (range 13-33%) of the labelled iodine, thyroxine containing 6% of thyroidal label. In plasma, equilibration was slower: PBI was 5% (range 2-10%) of plasma I-125 after 1 month, 11% (6-15%) after 2 months, 22% (10-33%) after 3 months, and 37% (14-62%) after 4 months. Free thyroxine increased during the first months and was thereafter variable (usually 0.5-1.5% of the plasma label).

Total thyroxine concentration thus may be up to 50% of total iodine, 0.01 µg/ml, i.e. 0.1 ng/ml. Free thyroxine is about 0.1 ng/ml. So, while the total thyroxine is low compared to mammals, free thyroxine is about five times the normal human concentration. This may explain the short biological half-life for I 125 thyroxine found in six series of determinations in *B. bufo* (range 11.0-16.8).

## References

- CHAMBERLAIN, P. *Cell Int. C.A.B.S.* 1969 177-287  
CHAMBERLAIN, P. *Fortschr. Zool.* 1974. 22-99

## D 48

Activation of Neurons in the Cat *Medulla oblongata* That are Activated by Hind Limb Group I Muscle—and by Skin Afferents

By H. JOHANSSON and H. SÄLVENDEN, *Department of Physiology University of Umeå, Sweden*

A medullary relay of the hind limb group I path to the cerebral cortex is located in lamina II (Brodal and Pompeiano (1957), as shown by Lundgren and Sjöström (1971).

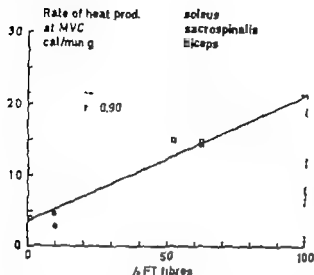


Fig. 1 Maximal rate of heat production in different muscles related to percentage of fast twitch (FT) fibres.

serted 5-6 cm into the muscle through a hypodermic needle. Provided no heat is lost from the monitored region the local heat production in the muscle can be calculated as  $\dot{Q} = \Delta T / \Delta t \cdot q$  cal/min g, where  $\Delta T / \Delta t$  is the temperature rise per minute and  $q$  is the specific heat of muscle estimated as 0.9 cal/g.

$\dot{Q}$  rose in all experiments with increasing force of contraction. The mean rate of heat production at MVC was 0.55 cal/min g (range 0.29-0.90  $n=11$ ) in soleus, 0.66 cal/min g (range 0.41-1.07  $n=6$ ) in sacrospinalis and 1.48 cal/min g (range 1.30-1.71  $n=5$ ) in biceps.

Muscle biopsies were taken from 14 muscles and fibre type distribution determined histochemically by staining for ATPase at pH 9.4.

The rate of heat production at MVC showed positive correlation to the area of fast twitch (FT) fibres given as percent of the cross-section area of the muscle (Fig. 1). The regression line is given by  $\dot{Q} = 0.018\% \text{ FT} + 0.35$   $r=0.90$ .

Linear extrapolation suggests that a muscle composed exclusively of FT fibres would have a maximal heat production of 2.15 cal/min g, whereas a muscle containing slow twitch (ST) fibres only would have a maximum of 0.35 cal/min g.

Thus, according to this estimate, the maximal energy turnover in human FT fibres is approximately six times that of ST fibres during voluntary isometric contraction.

#### Reference

GOLDSPENCK, G. R. E. LARSON and R. E. DAVIES, *Z. exp. Physiol.* 1970 66, 389-397.

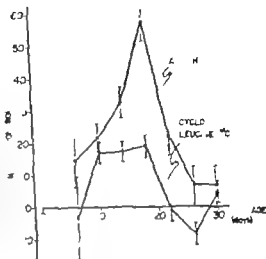
#### D-47

#### Metabolism of Iodine and Iodinated Thyroid Products in Amphibia

By P. ROSENKILDE, Zoophysiological Laboratory A University of Copenhagen, 15 Universitetsparken, Denmark

A survey of thyroid hormone dynamics in adult amphibia is attempted. The most complete investigation was made on the common toad *Bufo bufo*. I 131 uptake, total iodine

1. Effects of GH on accumulation of JB and  $^{14}\text{C}$ -cycloleucine of diaphragm normal rats of different ages. Hemidiaphragms were incubated for 90 min with and without GH (5  $\mu\text{g}/\text{ml}$ ) in Krebs-Henseleit bicarbonate buffer containing glucose (2.5  $\text{mg}/\text{ml}$ ), H-AIB (0.1 mM) and cycloleucine (0.1 mM), and the distribution of the labeled amino acids were measured at the end of the incubation period as described earlier (Hjølse and Ahrén, 1967a). Values presented are pooled means  $\pm$  S.E. of 5 different experiments including diaphragms. The effect of GH is given as ratio of control.



in young normal rats showed marked sensitivity to the stimulatory effect of GH. Studies have therefore been started to elucidate the sensitivity to GH of diaphragms from young normal rats of different ages. It can be seen from Fig. 1 that GH increases the uptake of non-utilizable amino acids AIB ( $\alpha$ -aminoisobutyric acid) and cycloleucine in diaphragms from 10, 14, and 18 day old rats; the effect being most pronounced in diaphragms from 18 day old rats. No significant stimulatory effect of GH was noticed in diaphragms from 6, 14, and 30 day old rats. Thus, the present results demonstrate that the diaphragm muscle from normal rats is considerably sensitive to GH when excised from rats of certain ages. At present there is no clear explanation to the changes in sensitivity to GH in relation to age, but the present working hypothesis is that the changes in sensitivity might be due to altered levels of endogenous secretion of GH paralleling the changes in feeding habits when the rats grow older.

### References

- HJØLSE, A. and K. ÅHRÉN, *Acta endocr. (Kbh.)* 1967, 34, 643-662.  
HJØLSE, A. and K. ÅHRÉN, *Acta endocr. (Kbh.)* 1967A, 36, 347-358.

### D 50

#### Three-component Output Analysis of Respiration during Rebreathing and Exercise in Man

By S. AND STEN AND PEDERSEN, MICHAEL GRUNSTEIN, JAKOB MERTZ, JOSEPH MILLONIGLI and POUL ERIK PAULSEN, *Institute of Medical Physiology B, University of Copenhagen, Denmark and Department of Physiology, McGill University, Montreal, Canada*

The  $\text{CO}_2$  sensitivity of the respiratory control system has usually been referred to the increment in ventilation ( $V_E$ ) per increment in  $\text{P}_{\text{CO}_2}$ . The results concerning  $\text{CO}_2$  sensitivity

Recent findings further demonstrate that medullary axon-collaterals of the dorsal spinal cerebellar tract, DSCT monosynaptically activate group I relay cells in nucleus Z (Johansson and Silfverius 1973). Extended observations do however show that ipsilateral group I hind limb afferents also excite medullary neurones which presumably are not located in nucleus Z.

Recordings have been made from 13 group I and 3 skin activated cells, localized laterally to nucleus Z, and from 6 group I and 2 cutaneous cells found rostralateral to the nucleus. The properties of the cells recorded from were similar to those of the nucleus Z. They were thus medullo-thalamic relay cells found among the neurones with the locations mentioned. Evidence for DSCT axon-collateral activation of these group I relay cells was also found.

The results suggest that the cells localized lateral to nucleus Z are located in the rostral portion of the cuneate nucleus, and that the cells localized rostralateral to nucleus Z belong to the hitherto functionally undefined nucleus X of Brodal and Pompeiano (1957).

DSCT would thus provide short latency information to a medullary nucleus classically considered to forward forelimb information (cuneate nucleus) and to a nucleus (X) anatomically known to project to the posterior cerebellum (Brodal and Torvik 1957). An anatomical confirmation of a hind limb input to the rostral portion of the cuneate nucleus would exclude the possibility that the unitary observation made is due to recordings from aberrant neurones.

### References

- BRODAL, A. and O. POMPEIANO, *J. Amer.* 1957 91 438-454  
 BRODAL, A. and A. TORVIK, *Arch. Psychiat. Nervenk.* 1957 195 550-567  
 JOHANSSON, H. and H. SILFVERIUS, *Acta ph. med. scand.* 1973 Suppl. 396. 94  
 LANDGREN, S. and H. SILFVERIUS, *J. Physiol.* 1971 18 551-571

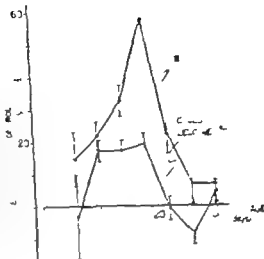
### D 49

#### Sensitivity to Growth Hormone of Diaphragm Muscle from Young Normal Rats

By K. ALBERTSSON WIKLAND and O. ISAKSSON, Department of Physiology, University of Göteborg, Sweden

By few exceptions the metabolic effects and the mechanism of action of growth hormone (GH) have been studied in hypophysectomized animals. GH administered *in vivo* or *in vitro* has a stimulatory effect on several metabolic events, like protein synthesis and the membrane transport of amino acids and sugars in different tissues from hypophysectomized animals. The early stimulatory effect of GH in e.g. diaphragms from hypophysectomized rats is after approximately 3 h followed by a "late inhibitory" effect of the hormone (Hjalmarsson and Ahren 1967 b). However other hormones than GH are also eliminated by hypophysectomy. This opens the possibility that the effects of GH in hypophysectomized animals do not reflect the physiological function of the hormone. Only small stimulatory effects or no effects at all have previously been observed in isolated tissues from normal rats. In preliminary experiments, however we observed that the diaphragm

Effects of GH on accumulation of  $^{14}\text{C}$ -labeled amino acids of diaphragm muscle of different ages. Hemidiaphragms were incubated for 90 min with and without GH (5  $\mu\text{g}/\text{ml}$ ) in Krebs-Henseleit buffer containing glucose (5  $\mu\text{g}/\text{ml}$ ),  $\text{pH}$ -7.4 (10.1 mM) and glucose (0.1 mM), and the distribution of the labeled amino acids were measured at the end of the incubation period. Values presented are pooled means of 5 different experiments including phrenic. The effect of GH is given out of control.



young normal rats showed marked sensitivity to the stimulatory effect of GH. Studies therefore been started to elucidate the sensitivity to GH of diaphragms from young rats of different ages. It can be seen from Fig. 1 that GH increases the uptake of non-utilizable amino acids AIB ( $\alpha$ -aminobutyric acid) and cycloleucine in diaphragms 10, 14, and 18 day old rats, the effect being most pronounced in diaphragms from 18 day old rats. No significant stimulatory effect of GH was noticed in diaphragms from 6, 22 and 30 day old rats. Thus, the present results demonstrate that the diaphragm muscle of young rats is considerably sensitive to GH when excised from rats of certain ages. At present there is no clear explanation to the changes in sensitivity to GH in relation to age, the present working hypothesis is that the changes in sensitivity might be due to altered rates of endogenous secretion of GH paralleling the changes in feeding habits when rats grow older.

### References

- WILSON, A. and K. ARVID, *Acta endocr* (Kbh) 1967a, 54, 645-662.  
WILSON, A. and K. ARVID, *Acta endocr* (Kbh) 1967b, 56, 347-358.

### D 50

#### ree-component Output Analysis of Respiration during Rebreathing and Exercise in

By Søren STENYANG PEDERSEN, MICHAEL CRUNTEIN, JAKOB MERTZ, JOSEPH MILIC-EMILI and POUL-ERIK PAULSEN. Institute of Medical Physiology B, University of Copenhagen, Denmark and Department of Physiology McGill University Montreal, Canada

The  $\text{CO}_2$  sensitivity of the respiratory control system has usually been referred to the change in ventilation ( $V_E$ ) per increment in  $P_{a\text{CO}_2}$ . The results concerning  $\text{CO}_2$  sensitivity

Recent findings further demonstrate that medullary axon-collaterals of the dorsal spinocerebellar tract DSCT monosynaptically activate group I relay cells in nucleus Z (Johansson and Silfvenius 1973). Extended observations do however show that ipsilateral group I hind limb afferents also excite medullary neurones which presumably are not located in nucleus Z.

Recordings have been made from 13 group I and 3 skin activated cells, localized later to nucleus Z, and from 6 group I and 2 cutaneous cells found rostralateral to the nucleus. The properties of the cells recorded from were similar to those of the nucleus Z. They were thus medullo-thalamic relay cells found among the neurones with the locations mentioned. Evidence for DSCT axon-collateral activation of these group I relay cells was also found.

The results suggest that the cells localized lateral to nucleus Z are located in the rostral portion of the cuneate nucleus, and that the cells localized rostralateral to nucleus Z belong to the hitherto functionally undefined nucleus X of Brodal and Pompeiano (1957).

DSCT would thus provide short latency information to a medullary nucleus classically considered to forward forelimb information (cuneate nucleus) and to a nucleus (X) anatomically known to project to the posterior cerebellum (Brodal and Torvik 1957). An anatomical confirmation of a hind limb input to the rostral portion of the cuneate nucleus would exclude the possibility that the unitary observation made is due to recordings from aberrant neurones.

### References

- BRODAL, A. and O. POMPEIANO, *J. Amer.* 1957 91 438-454  
 BRODAL, A. and A. TORVIK, *Arch. Psychiat. Nervenk.* 1957 195 550-567  
 JOHANSSON, H. and H. SILFVENIUS, *Acta physiol. scand.* 1973 Suppl. 396. 94  
 LANDGREN, S. and H. SILFVENIUS, *J. Physiol.* 1971 218 551-571

### D 49

#### Sensitivity to Growth Hormone of Diaphragm Muscle from Young Normal Rats

By K. ALBERTSSON WIKLAND and O. ISAKSSON *Department of Physiology, University of Göteborg, Sweden*

By few exceptions the metabolic effects and the mechanism of action of growth hormone (GH) have been studied in hypophysectomized animals. GH administered *in vivo* or *in vitro* has a stimulatory effect on several metabolic events, like protein synthesis and the membrane transport of amino acids and sugars in different tissues from hypophysectomized animals. The early stimulatory effect of GH in e.g. diaphragms from hypophysectomized rats is after approximately 3 h followed by a "late inhibitory" effect of the hormone (Hjalmarsson and Ahren 1967 b). However other hormones than GH are also eliminated by hypophysectomy. This opens the possibility that the effects of GH in hypophysectomized animals do not reflect the physiological function of the hormone. Only small stimulatory effects or no effects at all have previously been observed in isolated tissues from normal rats. In preliminary experiments, however we observed that the diaphragm



ventilation is the product of frequency and volume, and opposing changes in the two may explain the above discrepancy.

**2) Volume ( $V_T$  or  $V_{TM}$ ), frequency ( $f$ ) and ventilation ( $\dot{V}_E$ )** was measured in 6 subjects rebreathing from a bag initially filled with 6-8 l of 7%  $\text{CO}_2$  in 50%  $\text{O}_2$  at different steady levels of cycling (600, 1200 and 1800 kpm/min). The data was recorded and controlled on-line breath-by-breath with a system described earlier (Paulsen 1971).

**3) Volume control.** In all the subjects the increment in  $V_{TM}$  per increment in  $P_{ET\text{CO}_2}$  ( $\Delta V_{TM}/\Delta P_{ET\text{CO}_2}$  or slope) during rebreathing was largest at rest and gradually falling at work rates (fig. 1). **The time control.** The increment in frequency ( $\Delta f$ ) per increment in  $P_{ET\text{CO}_2}$  ( $\Delta f/\Delta P_{ET\text{CO}_2}$ ) during rebreathing was smallest at rest in all subjects, rising more and more following higher work rate. **Ventilation.** The ventilatory output varied unsteadily in the subjects, some showing an unchanged sensitivity ( $\Delta \dot{V}_E/\Delta P_{ET\text{CO}_2}$ ) to  $\text{CO}_2$  during rebreathing at higher levels. Other subjects showed a decreasing  $\Delta \dot{V}_E/\Delta P_{ET\text{CO}_2}$  from rest to the highest work rates. The peak value of the first time derivative of flow ( $A_1$ ), the non-volume related respiratory output was measured, and shows a curvilinear relation with increasing slope at increasing work rates (Fig. 1). This parameter is used within the first 100 ms of inspiration, and thus  $A_1$  is only slightly dependent upon work rate.

The rate of change in  $P_{ET\text{CO}_2}$  remained constant in spite of the ever increasing  $\dot{V}_E$ , which indicates a stable relationship between  $P_{ET\text{CO}_2}$  and medullary chemoreceptor  $P_{\text{CO}_2}$ , thus indicating that  $P_{ET\text{CO}_2}$  may be as valid an input index for the  $\text{CO}_2$ -stimulus as the arterial  $P_{\text{CO}_2}$ .

The above mentioned conflicting reports concerning  $\text{CO}_2$  sensitivity expressed as ( $\Delta \dot{V}_E/\Delta P_{\text{CO}_2}$ ) during exercise may thus be an unnecessarily complicated concept, due to opposing changes in the 2 factors ( $f$  and  $V_{TM}$ ). Clarification of the problem requires separate measurements of the pacemaker and volume regulating system.

### References

- BRUNN, E. and M. NIELSEN, *Acta physiol. scand.* 1957 39 27-35.
- MCNAB, A. B. JR. and S. A. BARCOCK, *J. appl. physiol.* 1962, 17 674-676.
- PAULSEN, J. B., W. W. PETER, E. B. PAGE and R. J. ATWELL, *J. clin. Invest.* 1951 30, 503-516.
- THORNTON, J. P. J., *U.S. Air Force Tech. Rep.* 1951 6328 479-485.
- ULFENDARF, H. R. *J. appl. Physiol.* 1971 30 165-172.

D 51

### Kinetics of the Glomerular Ultrafiltration

By Ö. KÄLLÉKÖ, L.-O. LINDBOM, H. R. ULFENDARF and M. WOLGAST *Department of Physiology and Medical Biophysics, Biomedical Center University of Uppsala, Sweden*

A lot of problems are still to be solved about the kinetics of the glomerular filtration process. According to the results of Brenner and coworkers the general opinion seems to be that the filtration process is in equilibrium in the glomerulum. This should mean, that the net driving force at the end of the glomerular capillary is zero. The results of the present investigation show however a considerable distance from equilibrium.

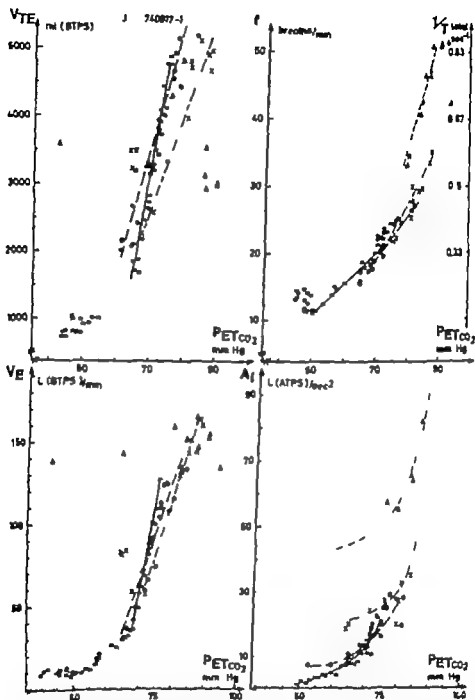


Fig. 1 The CO<sub>2</sub> response curves for expired tidal volume ( $V_T$  or  $V_{Tm}$ ), respiratory frequency ( $f$ ), ventilation ( $V_E$ ) and inspiratory peak acceleration ( $A_1$ ). The symbol  $\Delta$  denotes rebreathing during rest and the solid lines are tentative slopes during this condition. Rebreathing during steady state cycling is symbolized with  $\circ$ ,  $\times$ ,  $\Delta$  for 600, 1200, and 1800 kpm/min, respectively. The related slopes and curves are: Dashed, short and long dashes, and small dashes.

during exercise have been diverging, some finding increased sensitivity (Hickam, Pryor Page and Atwell 1951), some finding decreased sensitivity (Muxworthy 1951), while others found it to be unchanged (Asmussen and Nielsen 1957, Craig and Babcock 1962).



The experiments were performed on adult Sprague Dawley rats and with micropuncture techniques the single nephron glomerular filtration rate, the filtration fraction and oncotic and hydrostatic pressures in superficial nephrons and vascular structures were measured and calculated. The mean net driving pressure was about 20 mm Hg and the driving pressure at the end of the glomerular capillary was about 13 mm Hg. From experimental values it could mathematically be shown, that the glomerular filtration must not by necessity be correlated to the glomerular plasma flow as postulated by Brenner. Rather it seems to be a combined influence of hydrostatic and oncotic pressures within the glomerulum and the glomerular plasma perfusion rate. According to our results pre- and post-glomerular resistances regulate these parameters.

### Reference

- BRENNER, B. M., W. M. DUM and C. R. ROBERTSON, *The physiological basis of glomerular ultrafiltration*. In *Kidney and urinary tract physiology* (ed. U. Thurnau). London 1974. P. 336.





# ACTA PHYSIOLOGICA SCANDINAVICA

VOLUME 99

NUMBER 1

January 1977

646

SS  
7-5-77

Contents

- 1 Rutå, G. and K. E. Arfors  
Protein Concentration in Interstitial and Lymphatic Fluids from the Subcutaneous Tissue
- 9 Bäckström, M., L. Olsson and A. Seliger  
Protein Secretion Metabolism in Non-Innervated Perineal Glands before and after Intra-ocular Malignancy: Supersensitivity of Adrenoceptors that have never been innervated
- 19 Heurteaux, O. and P. Sejersten  
Local Reflex in Microcirculation in Human Skeletal Muscle
- 27 Ross, S. B. and M. Kalder  
Efflux of 5-Hydroxytryptamine from Synaptosomes of Rat Cerebral Cortex
- 37 Lindqvist, L., O. Nilsson and G. Ronquist  
Preferential Uptake of  $^{14}$ C- $\alpha$ -Aminoisobutyric Acid into Mouse Uterine Tissue during Early Pregnancy
- 42 Huberman, D., C. H. Håkansson, U. Merzke and N. G. Tonnesen  
Intra- and Extracellular Activity of Calbindin Cells
- 49 Ekström, J.  
The Effect of Tooth Amputations on the Choline Acetyltransferase Activity of Rat Sub-mandibular Glands
- 53 Lönnerholm, G.  
Carbonic Anhydrase in the Intestinal Tract of the Guinea-pig
- 62 Holmgren, E.  
Regulation of the Heart of *Tetraodon lineatus* by Autonomic Nerves and Circulating Catecholamines
- 75 Berglindh, T.  
Potentiation by Carbachol and Aminophylline of Histamine- and db-cAMP Induced Parietal Cell Activity in Isolated Gastric Glands
- 85 Ahlström, E.  
Regulation of Respiration: "The Black Box"

## Editorial Board

P Kruhoffer  
København

K. Hartala  
Turku

J Jansen  
Oslo

Y Zotterman  
Stockholm

U S von Euler  
(Editor) Stockholm

## Editorial office

Acta Physiologica Scandinavica  
Karolinska Institutet  
S 104 01 Stockholm

The "Acta physiologica scandinavica" are published for the Scandinavian Society for Physiology and contain contributions to Physiology Medical Chemistry or Pharmacology by Scandinavian authors or from Scandinavian laboratories. The articles are published in English, French or German. Each number consists of about 9 printed sheets, 4 numbers forming a volume. Not more than 3 volumes will appear each year. Subscriptions should be mailed to Acta Physiologica Scandinavica, Karolinska Institutet, S 104 01 Stockholm, Sweden.

Price per volume 120 Sw Cr

Manuscripts from Denmark should be sent to Professor P Kruhoffer  
Med. Fysiologiska Inst., Universitetet, Julevej 30, Dk 2100 København Ø

Manuscripts from Finland should be sent to Professor K. Hartala,  
Fysiologiska Institutionen, Universitetet, Turku

Manuscripts from Norway should be sent to Dr med. Jan Jansen,  
Fysiologisk Institutt, Universitetet Karl Johans Gste 47 Oslo, and

Manuscripts from Sweden should be sent to Professor Y Zotterman,  
Wenner Gren Center Konferenssekretariatet, 23 tr Sväsvägen 168, 113 46 Stockholm.

DR ROBERT HEILIG LIBRARY  
S.M.S. MEDICAL COLLEGE

JAIPUR 27992

Acc. No. 811104

Date

Price subs



# ACTA PHYSIOLOGICA SCANDINAVICA

## Editorial Board

P. Ernerfelt  
København

K. Hartale  
Turku

J. Jansen  
Oslo

Y. Zolnerman  
Stockholm

U. S. von Euler  
(Editor) Stockholm

## Vol. 99 INDEX

Part. 1. (January 1977)

Protein Concentration in Interstitial and Lymphatic Fluids from the Subcutaneous Tissue. By O. RUTELJ and K.-E. AARFÖD	1
Pinac Serotonin Metabolism in Non-Innervated Peritoneal Glands before and after Intracocular Maturation. Super-sensitivity of Adrenoceptors that have never been innervated. By M. BLACKSTADT, L. OLSSON and Å. SELLER	9
Local Reflex in Microcirculation in Human Skeletal Muscle. By O. HENRIKSSON and P. SÖDERM	19
Effect of 5-Hydroxytryptamine from Synaptosomes of Rat Cerebral Cortex. By R. B. ROSS and D. KILB	27
Preferential Uptake of <sup>14</sup> C- $\alpha$ -Aminobutyric Acid into Mouse Uterine Tissue during Early Pregnancy. By I. LINQVIST, O. NILSSON and G. RÖNQVIST	37
Intra- and Extracellular Activity of Ciliated Cells. By D. HUMERMAN, C. H. HANSSON, U. VIERCKE and N. G. TORAMANN	42
The Effect of Teeth Amputations on the Choline Acetyltransferase Activity of Rat Submandibular Glands. By J. EKLUND	48
Carbonic Anhydrase in the Intestinal Tract of the Elephant. By G. LÖNNERSTAM	53
Regulation of the Heart of a Teleost, <i>Gasterosteus aculeatus</i> , by Autonomic Nerves and Circulating Catecholamines. By S. HÖRRELL	62
Prostaglandin by Carbachol and Aminophylline of Histamine and db-cAMP-Induced Parietal Cell Activity in Isolated Gastric Glands. By T. BERGSTRÖM	75

Regulation of Respiration "The Black Box" By E. ASMUSSEN

Time Course of Changes in Human Skeletal Muscle Succinate Dehydrogenase and Cytochrome Oxidase Activities and Maximal Oxygen Uptake with Physical Activity and Inactivity By J. HENRIKSSON and J. S. REITMAN

The Effect of Plasma Proteins on the Capillary Permeability in the *rete mirabile* of the Eel (*Anguilla vulgaris* L.). By K. J. MYRNE and J. B. STEEN

Skeletal Muscle Fiber Splitting Induced by Weight Lifting Exercise in Cats. By W. GONZALEZ, E. C. ERICSON and F. BONDE PETERSEN

Choline Acetyltransferase Activity in Parotid Glands of Rats after Prolonged Treatment with Pilocarpine. By J. EKSTRÖM

Influence of Acetate on the Metabolism of  $\beta$ -Hydroxybutyrate in the Perfused Hind-Quarter of the Rat. By N. KARLSSON, E. FELLENIUS and K. H. KILSSON

### Short Communications.

Training Induced Changes in the Subgroups of Human Type II Skeletal Muscle Fibres. By P. ANDERSEN and J. HENRIKSSON

Vagal Release of Somatostatin into the Antral Lumen of Cats. By K. UYKAS-WALLINSTEIN, S. ELENIC and R. LUTT

### Part 2 (February 1977)

Conservate-like Membrane Structures and Olfactory Transduction. By G. O. SPERBER

Twenty Four Hour Variation in Flow and Composition of Leg Lymph in Normal Men. By A. ENGSET, W. OLSEWICKI, P. M. JÄGER, J. SOKOLOWSKI and L. THODORSEN

Flow and Composition of Leg Lymph in Normal Men during Venous Stasis, Muscular Activity and Local Hyperthermia. By W. OLSEWICKI, A. ENGSET, P. M. JÄGER, J. SOKOLOWSKI and L. THODORSEN

Influence of Acetate on the Metabolism of Palmitate in the Perfused Hind-Quarter of the Rat. By N. KARLSSON, E. FELLENIUS and K. H. KILSSON

Contractile Responses in Noradrenaline Varying Dependence on External Calcium of Consecutive Vascular Segments of Perfused Rat Hindquarters. By M. C. SUTTER, M. HALLBÄCK, J. V. JONES and B. FOLKOW

Time Course and Extent of Carotid Sinus Baroreceptor Threshold Resetting in Rats with Renovascular Hypertension. By J. V. JONES

Rate-dependent Myogenic Response of Vascular Smooth Muscle during Imposed Changes in Length and Force. By S. B. SJÖURDSSON, B. JOHANSSON and S. MELLANDER

Localization of Adrenergic Receptors in Guinea Pig Ileum and Rabbit Jejunum to Cholinergic Neurons and to Smooth Muscle Cells. By J. WIKBERG

Cardiovascular Control in the Milan Strain of Spontaneously Hypertensive Rat (MHS) at "Rest" and during Acute Mental "Stress" By M. HALLBÄCK, J. V. JONES, G. BIANCHI and B. FOLKOW

Conjugated Catechol Derivatives in a Transplantable Islet Cell Tumour of the Golden Hamster. By D. FALCK, C. HANSSON, B. M. KENNEDY and E. RÖNNQVIST

Actomyosin ATPase, Myokinase, CPK and LDH in Human Fast and Slow Twitch Muscle Fibres. By A. THORSTENSSON, B. SÖÖN, P. TIECH and J. KARLSSON

Lactate in Fast and Slow Twitch Skeletal Muscle Fibres of Man during Isometric Contractions. By P. TIECH and J. KARLSSON

Effect of Changes in Blood Volume Distribution on Circulatory Variables and Plasma Renin Activity in Man. By S. BEVEGÅRD, J. CASTENIERS and L. E. LINDBLAD

Effects of Carbachol and Calcium on the Cyclic Guanosine-3',5' Monophosphate (cyclic GMP) Metabolism in Intestinal Smooth Muscle. By K. B. NILSSON and R. G. G. ANDERSSON

## Short Communications

Metaplastic Effect of Adenosine in Dog Adipose Tissue <i>in Situ</i> . By B. B. FRIEDHOLM and A. SOLLENI	254
Part. 3. (March 1977)	
Effect of Oxygen on Peripheral Airways. By T. M. NIELSEN and O. F. PEDERSEN	257
Cardio-Haemodynamics in the Perinatal Period. A Study in Lambs. By A. APFEL, O. BROSTROM, P. HENRIK and L. JOHANSSON	261
Regional Distribution of the ECG in the Rat: Variation with Odour Quality. By G. THOMASSEN and K. B. DOVING	270
Effects of Acetylcholinesterase on Alkalinization, Acid Secretion and Electrogenic Properties in the Isolated Gastric Mucosa. By A. GARNER	281
Receptor Fibres to the Tongue in the Chorda Tympani Proper Nerve. By G. HELLEKANT	292
Blood Pressure and Heart Rate Regulating Capacity of the Carotid Sinus during Changes in Blood Volume Distribution in Man. By S. BRUNZELAND, J. CASTENFORS, L. E. LINDHOLM and J. TRANSD	300
Changes in ATP and Cyclic Nucleotide Levels during Sympathetic Nerve Stimulation in Canine Subcutaneous Adipose Tissue <i>in situ</i> . By B. B. FRIEDHOLM, E. BELFRAGE and E. BLASCHKE	313
Renal Na <sup>+</sup> -ATPase Activity during Saline Infusion in the Rabbit. By O. M. SEIERSTED	323
Effect of Sodium Pentobarbital on the Apparent Turnover of Acetylcholine in Different Brain Regions. By A. NORDBERG and A. SUNDBALL	334
Adrenergic and Cholinergic Nerves of the Human Urethra and Urinary Bladder: A Histochemical Study. By A. EK, P. ALM, K. E. ANDERSSON and C. O. A. PETERSON	345
Lower Body Negative Pressure and Effects of Autonomic Heart Blockade on Cardiovascular Responses. By H. BRUNSTED, P. ROMANOWSKI and G. TYDEN	353
Slow Action of Ca on Myelinated Nerve Fibres of <i>Xenopus laevis</i> . By T. BILZMAR	361
The Effects of Variations in Extracellular Magnesium Concentration on Electrical and Mechanical Activity in Rat Portal Vessels. By S. B. SORUMTSOV and B. UVELLUS	368
Quantitative Evaluation of the Blood-Brain Barrier Capacity to Form Dopamine from Circulating L-DOPA. By J. E. HARDING, I. EDVINGSON, CH. O'BRIEN and E. ROSENDAHL	377
Part. 4. (April 1977)	
The Compliance Curve for the Flow Limiting Segments of the Airway: I. Model Studies. By O. F. PEDERSEN and T. M. NIELSEN	385
Effect of the Polyene Antibiotic Filipin on the Permeability of the Inward- and the Outward-facing Membranes of the Isolated Frog Skin ( <i>Rana temporaria</i> ). By R. NIELSEN	399
Extracellular Potassium Concentration in Juvenile and Adult Rat Brain Cortex during Apnoea. By A. J. HANSEN	412
Distribution of Coronary Blood Flow in the Left Ventricular Wall of Dogs Evaluated by the Uptake of Xe 133. By H. BAAGER	421
Uptake for Rate-Sensitive Regulatory Mechanism in Myogenic Microvascular Control. By P.-O. GRAND, J. LUNDVALL and S. MELLANDER	432
Characteristics of Aortic Baroreceptor C-Fibres in the Rabbit. By P. THOREN and J. V. JONES	448
Alpha-Receptor Subsensitization of Isolated Atria from Rats Following Physical Training or Repeated ACTH-Injections. By A. SALONVUO, R. TERRI and M. N. E. HARRIS	457
Effect of Hypoxia on Mitochondrial Mass and Cytochrome Concentrations in Rat Heart and Liver during Postnatal Development. By V. L. KIVALLA and I. HANSEN	462
The Effect of 6-Aminocaproic Acid Blockade of the Pentose Phosphate Pathway on Catecholamines in the Rat Adrenal Medulla, Superior Cervical Ganglion, Hypothalamus and Synaptosomal Fractions. By S.-E. JENSEN, J. GRIPENBERG and M. HILANDER	467

- The Ability of ATP Free Granule Material from Bovine Adrenal Medulla to Bind Inorganic Cations and Biogenic Amines. By B. UVNÄS and C. H. ÅBERG
- Contractility Muscle Mass and Agonist Sensitivity of Isolated Portal Veins from Normo- and Hypertensive Rats. By M. C. SUTTER and B. LJUNG
- Effect of Atropine and Methamidate on Vagally Induced Gastric Acid Secretion and Gastrin Release in Anesthetized Cats. By K. UVNÄS-WALLÉN and H. ANDERSSON
- Effect of Hypercapnia and Hypocapnia on Tryptophan and Tyrosine Hydroxylation in Rat Brain. By A. CARLSSON, T. HOLMÉN, M. LINDQVIST and B. K. SÆVØ

*Short Communication.*

- Increase in Gastrin Concentration in the Duodenal Mucosa of Dogs Following Resection of the Gastric Antrum. By G. NILSSON and K. BRÖNN

*Supplements*

- Supplementum 442. The Renin Angiotensin System and Kidney Function. A Review of Contributions to a New Theory. By P. P. LEYSSAC.
- Supplementum 443. Muscle Strength, Fibre Types and Enzyme Activities in Man. By A. THORSSON.
- Supplementum 444. Sodium and Angiotensin II in the Central Control of Fluid Balance. A Study in conscious goats. By L. ERIKSSON.

## Protein Concentration in Interstitial and Lymphatic Fluids from the Subcutaneous Tissue

By

GIAMFRANCO RUTELLI and KARL E. ARFORS

Received 24 February 1976

### Abstract

RUTELLI, G. and K. E. ARFORS. Protein concentration in interstitial and lymphatic fluids from the subcutaneous tissue. *Acta physiol. scand.* 1977 99 1-8.

The protein content of interstitial fluid and lymph from the same tissue was determined, fluid samples of identical volumes being taken from the subcutaneous tissue of rabbits by micropunctures. The same size lymph as collected from lymphatic vessels of 30-100  $\mu$ m. An electrophoretic technique as polyacrylamide gels in glass capillaries as used for the analysis of proteins. The gels were stained and then viewed on microscope equipped with moving table and photomultiplier. The area under each protein band was calculated from the recorded densitograms. The method required only few asolites of fluid for analysis. Using solutions of known protein concentrations the relationship between the amount of protein and the area under the densitogram band as investigated. This relationship was found to be linear making it possible to quantify the protein content of both interstitial fluid and lymph samples. The latter fluid, plasma concentration ratios for albumin, transferrin, globulins and total proteins are 0.42, 0.42, 0.32 and 0.37. The albumin globulin ratio in interstitial fluid as found to be 1.40 of the plasma value although the values obtained for lymph were similar to those found for interstitial fluid. The similarity between the protein concentration of interstitial fluid and lymph indicated that the endothelial lining of the terminal lymphatics did not restrict the passage of macromolecules into the lymphatics.

Key words: Interstitial fluid, lymph, proteins, subcutaneous tissue, permeability

According to the classical Starling-Landis concept, capillary fluid balance is maintained by dynamic interaction between hydrostatic and osmotic forces acting across the capillary wall. These forces are considered to be of almost equal magnitude, therefore cancelling each other out. Efforts have been made during recent years to measure the numerical values of the forces, but Wiedersheim (1971) has pointed out that the literature on capillary fluid balance still contains a number of apparently conflicting results.

One of these conflicts results from the fact that while direct measurements of hydrostatic pressure (Richardson and Zwiefach 1970) have been made possible by sensitive micropressure devices (Isaia et al. 1970), the bulk of our information about interstitial fluid osmotic pressure is derived from measurement of lymph protein concentration. Experimental evidence that tissue fluid and lymph are identical in their protein composition is still lacking. Moreover in recent reports (Casley-Smith and Bolton 1973, Casley-Smith

1976) it has been suggested that as a result of lymphatic compression, lymph is ultrafiltrate through the endothelium, thus increasing the protein concentration of the lymph as compared to the interstitial fluid concentration.

Direct measurements of interstitial fluid protein concentration are difficult since minute amounts of fluid are available by sampling from connective tissue, and adequate analytical methods for protein determination in such small volumes have not been available until now. Indirect estimates of interstitial fluid protein concentration have been made by determination of the partition of protein between plasma and the tissue space (Bens *et al.* 1955, Walker *et al.* 1960, Studer and Potchen 1968). These measurements, however, yield results which are intrinsically an expression of the average body or organ protein concentration, thus making comparison with lymph protein concentration difficult.

In the present investigation a technique making it possible to analyse the protein content of samples in the nanolitre range has been used to determine the protein concentration both in interstitial fluid and lymph from the same tissue. The method consists of micropuncture for the collection of lymph and tissue fluid and an electrophoretic technique for quantitative analysis of proteins in nanolitre samples of fluid.

## Materials and methods

### *Protein determination*

The protein content of interstitial fluid, lymph and plasma was determined by the disc-electrophoretic method of Ornstein (1964) and Davis (1964). A micro-scale modification, and the practical details, have been extensively described by Sreeds (1969). Certain further modifications were, however, made before study. In brief, the electrophoresis was run in small glass capillaries of 3  $\mu$ l volume (Drummond Sci. Co. Broomfield, Penn., U.S.A.) with i.d. 200  $\mu$ m containing a spacer and a running polyacrylamide gel. The concentration used was 5% in the spacer and 20% in the running gel. These concentrations are double those used by Sreeds (1969) and resulted in increased resolution of the different proteins.

Ammoniumperoxydisulfate was used instead of riboflavin because it leads to more uniform cross-linking in the polyacrylamide. The pulsed voltage over the gel was kept at 175 V with a pulse rate of 500 Hz and a duty-cycle of 25%. After the run the gel was pushed out of the capillary and placed in a solution of 20% sulfosalicylic acid for 15 min. Treatment of the gel with sulfosalicylic acid was performed to make the binding of Coomassie Blue to the protein more stable. After staining for 15 min in a 0.2% solution of Coomassie Blue the gel was washed several times in a solution of acetic acid, methanol and distilled water (1:5:10) and stored for 1 h in the same solution.

A photomicrograph of the gel is shown in Fig. 1. A total of 15 protein bands were stained but only albumin, transferrin and  $\alpha_2$  macroglobulin identified. The optical density of the stained protein bands was measured in a LEITZ ORTHOLUX microscope, fitted with a photomultiplier (EMI KNOTT Type 6094 A, Knott Elektronik, Munich, West Germany). A logarithmic Integrator Recorder (Vitalone, N.V. Dieren, Holland) gave recordings of protein extinction. For measurement the gel was placed in a slit (ca. 0.4 mm) made in a brass plate fixed to a plexiglass plate. A cover glass was placed over the slit to keep the gel wet during measurement. The gel was scanned at 6 mm/min using a powered microscope table. An adjustable slit in the light path of the microscope permitted measurement of a specified width of the gel. A yellow filter (LEITZ 510) was also placed in the light beam. A densitogram is shown in Fig. 2.

To make quantitative measurements of the different proteins in the gel, the relationship between the area under the curve and the amount of protein was investigated with solutions of standard human serum proteins (Boehringerwerke AG, West Germany) of known concentrations. The volumes of standard solutions used varied between 1  $\mu$ l and 15.0  $\mu$ l.

### *Sampling of interstitial fluid and lymph*

The experiments were performed on 12 rabbits. The details of the micropuncture technique used for collection of interstitial fluid have been described previously (Rutili and Arfors 1976).



Fig. 1. Photomicrograph of polyacrylamide gel electrophoresis of an interstitial fluid sample after staining with Coomassie Blue. The volume of the sample was 8.4  $\mu$ l.

In summary after careful dissection of the epidermal layer of the rabbit hindleg, the subcutaneous tissue was exposed. In the dissected area the microcirculation was readily visible and lymphatics of 30 to 100  $\mu$ m diameter could occasionally be observed. Lymphatic vessels smaller than 30  $\mu$ m and terminal sacs could not be identified. Using a micromanipulator a small oil-filled glass pipette with a tip diameter of 8-18  $\mu$ m was inserted into the tissue and fluid collected by slight aspiration produced by withdrawing 4-5 ml of air with a syringe connected to the pipette holder. The sample was placed in a teflon cup filled with paraffin oil so that the droplet assumed a spherical shape and its volume could be calculated by measurements of its diameter. The whole sample was then removed and placed onto the polyacrylamide gel. After electrophoresis the gel was stained for its protein content. Since a total of 20 samples could be run in parallel the analysis was carried out simultaneously with 4 lymph samples, 4 interstitial samples, 4 plasma and 8 standards for each rabbit. The volume of the standard protein solution was determined in the same way as for the interstitial fluid samples.

### Results

The relationships between the amounts of albumin, transferrin, globulins and total proteins, and the areas under absorption curves obtained from standard solutions were investigated.

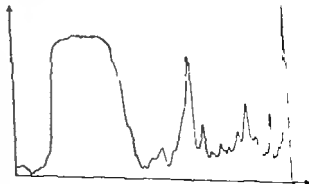


Fig. 2. Densitometer recording of the separation pattern of an interstitial fluid sample. The gel was scanned on a microscopie equipped with a slowly moving table and photomultiplier for measurement of the optical density.

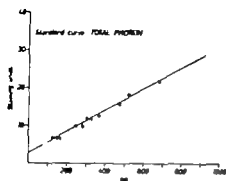


Fig. 3

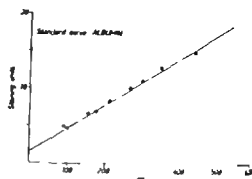


Fig. 4

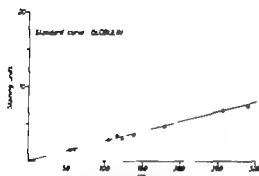


Fig. 5

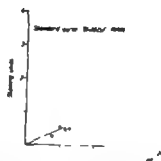


Fig. 6

Fig. 3 4 5 6. Relationship between the amount of Total Proteins, Albumin, Total Globulins, Transferrin and the amount of Coomassie Blue stain as measured (from the optical density of the stained protein bands). The amount of stain is expressed as Staining Units. (For more details see text.)

The results are shown in Fig. 3 4 5 and 6. The area under each curve is expressed in staining units, which are calculated for each protein fraction using the formula  $SU = I/s \cdot E$ .  $I$  is the number of integration impulses,  $s$  is the reading given by a standard reference signal for the chart recorder which varies with the sensitivity adjustment of the recorder and  $E$  is the fixed value of the standard signal (0.2).

An almost linear relation for the different proteins was found within the range used in this investigation and the coefficient of variation of the analytical procedure was calculated and found to be less than 12%. The protein concentrations of lymph (L) and interstitial fluid (IF) measured in the subcutaneous tissues of rabbits are given in Table I.

TABLE I. Plasma, lymph and interstitial fluid protein concentration (g/100 ml) of the subcutaneous tissue of rabbits. The values are mean  $\pm$  S.D. of 1 animals.

	Total protein	Albumin	Globulin	Transferrin
Plasma	6.63	3.62	2.68	0.7
S.D.	0.58	0.36	0.40	0.03
Lymph	2.66	1.81	0.82	0.11
S.D.	0.40	0.37	0.20	0.02
Int. fluid	2.45	1.60	0.86	0.11
S.D.	0.41	0.30	0.19	0.02



Interstitial fluid and lymph/plasma proteins. Concentration ratio of rabbit subcutaneous tissue.

	Tot protein	Albumin	Globulins	Transferrin	A/G
1	0.37	0.42	0.32	0.42	1.60
	0.06	0.08	0.07	0.09	0.22
	0.38	0.46	0.30	0.41	1.70
	0.06	0.08	0.09	0.08	0.70

significant difference between lymph and interstitial fluids was observed within the 1 error of the method.

Table II the protein concentrations of LF and lymph are compared with the respective plasma values.

values for the albumin/globulin (A/G) of Table II are also expressed as ratios of the values. No difference was found between LF and lymph.

similarity between interstitial fluid and lymph is shown more directly in Table III.

as for albumin, globulins and transferrin determinations of the single experiment given as a mean ratio between interstitial fluid and lymph concentration.

check the reliability of the micro-electrophoretic method for quantitative determination of proteins, plasma samples from 6 rabbits were analysed for their protein content by micro-zone electrophoretic standard method. The albumin/globulin ratios (A/G) obtained

with the micro-zone electrophoretic method (I) were 1.40, 1.44, 1.25, 1.56, 0.81, 0.98 those obtained with the micro-disk electrophoretic method (II) were 1.41, 1.50, 1.15, 0.92, 1.00 respectively. The ratio I/II was  $0.99 \pm 0.06$ .

### Discussion

electrophoretic method used in this investigation for both qualitative and quantitative analysis has provided a means of measuring the protein concentrations of body fluids in nanolitre amounts. The relatively high coefficient of variation of the method should be considered in relation to the small volumes of the samples used for protein analysis (1-15 nl). Comparatively, analysis of the same samples by an independent method

showed no significant difference between the methods and confirmed the validity of the micro-electrophoretic technique. As the relationship between staining units and protein concentration was linear quantitative determinations of protein concentrations in interstitial fluid, lymph and plasma were possible. The validity of the micropuncture sampling technique for the collection of tissue fluid has been discussed elsewhere (Ruttili and Ar

II) Albumin, globulins and transferrin interstitial fluid to lymph concentration ratio. The values are mean  $\pm$  S.D. of twelve rabbits.

	Globulins	Transferrin
12	$1.09 \pm 0.21$	$1.02 \pm 0.17$

The value of each single rabbit was obtained from 2 to 4 interstitial and lymph fluid samples.

fors, 1976) The overall findings of the study are consistent with the view that the samples collected are of interstitial fluid. The time required for dissection of the epidermal layer; collection of fluid samples ( $\approx 5$  min) is relatively short, and changes due to trauma and inflammation are minimal.

The concentration of total protein in the interstitial fluid and lymph from the rat subcutaneous tissue was found to be lower than the concentration in plasma (1.3). The values found are in agreement with those previously reported for the same tissue and species. Haljam  and Freden (1970) found an interstitial fluid to plasma protein of 0.32 and C t e (1960) a lymph to plasma protein ratio of 0.41.

Using nylon wicks to collect interstitial fluid, Aukland and Fadnes (1973) found an interstitial fluid to plasma protein ratio of 0.56 in rats which is somewhat higher than the value found in this investigation. Our lower values may be due to a species difference, studies where interstitial fluid collected using wicks were directly compared with interstitial fluid collected by micropuncture (Rutili and Arfors, to be published), no significant difference was found between the two methods.

Of the 15-16 different proteins stained, only the albumin, transferrin and total globulin concentrations were determined separately.  $\alpha_2$ -Macroglobulin could not be quantified accurately because of its low concentration. The concentrations of these protein fractions in the interstitial fluid and in the lymph were identical (Table I). The similarity in composition between the two fluids suggests that the passage of macromolecules from the tissues to the lymphatics is not restricted by the endothelial lining of the terminal lymphatics. The morphological explanation of this "no-restriction" concept was given by Leak and Burke (1968) and by Cliff and Nicoll (1970). In electron microscopic studies these workers have found "flaps and gaps" to be present in the endothelial layer of the terminal lymphatics permitting the passage of quite large molecules. Moreover they found anchoring filaments connecting the small lymphatics to the surrounding tissue, representing the mechanism responsible for opening the lymphatic capillaries in conditions of increased tissue pressure. The lack of a concentration gradient between the tissue fluid and the lymph fluid also indicates that the net transport of macromolecules between the two compartments is, at steady-state, a convective rather than a diffusive process. If this is a valid argument it will follow that the concentration of proteins in the tissue is, within their volume of distribution, evenly distributed. The capillary wall will thus represent the site of selectivity to molecular transport and the steady-state lymph/plasma ratio a measure of its permeability. Garlick and Renkin (1970) arrived at a similar conclusion as a result of kinetic studies of the passage of different macromolecules from blood to lymph. The hypothesis that lymph protein concentration is higher than interstitial fluid concentration (Casley-Smith and Bolton 1973; Casley-Smith 1976), providing the driving force for osmotic flow from interstitial fluid into lymphatic capillaries, is not supported by the present results. The idea that net transport of proteins from interstitial space into lymph by an osmotic flow occurring as a result of a concentration gradient of the same proteins, has already been shown to be incompatible with the physical laws of osmotic flow (Michel 1974).

The albumin/globulin (A/G) ratio in tissue fluid and lymph was found to be higher than the A/G ratio of plasma by a factor of 1.6-1.7. The absolute values of the A/G ratio

interstitial fluid and lymph are not given in the results because of the large variation in the A/G ratio in plasma between different rabbits (Table III). The mean A/G ratio in plasma was, however 1.35 compared to mean A/G ratio in tissue fluid of 2.20. A similar tissue fluid value (2.67) was found in the same tissue by Haljam  and Freden (1970).

A higher A/G ratio of interstitial fluid relative to plasma indicates a higher permeability of capillary walls to albumin than to globulins. The transport of proteins across the microcirculation is, according to Lassen et al. (1974) a unidirectional process. The functional morphological structure in the capillary walls through which this process takes place has been described by Grofte (1956). From studies of the transcapillary passage of dextran of various molecular size, he suggested the presence in the capillary wall of pores of 35-45 Å radius, excluding molecules of the size of albumin, and fewer large pores of 170-350 Å radius through which the filtration of plasma could occur.

The effective filtration area of porous membranes available for a molecule is a function of the effective molecular radius ( $R_{\text{eff}}$ ) and of the pore radius. Equations for the calculation of the effective filtration area have been formulated by Renkin (1954). Using these equations and assuming a  $R_{\text{eff}}$  for albumin of 35 Å and a  $R_{\text{eff}}$  for globulins of 50 Å, the A/G ratio found in these experiments may be explained in terms of filtration through pores of 250-300 Å diameter in the capillary wall.

An alternative to the large pore hypothesis is vesicular transport. Endothelial vesicles are firstly described by Palade (1953) after electron microscopic observation of the fine structure of the capillary wall. Electron dense tracers such as Ferritin ( $R_{\text{eff}}$  110 Å) or Gold ( $R_{\text{eff}}$  100 Å) injected into the circulation were found to be localized within the vesicles (Karnovsky 1968) suggesting their participation in transporting material across the capillary wall. Vesicular transport is subject to steric restriction (Garlick and Renkin 1970). Assuming a vesicle radius of 250 Å (Brune and Palade 1968, Casley-Smith 1968) globulins will be restricted by a factor 1.2-1.3 relative to albumin. This explains only in part the measured A/G ratio of 1.6-1.7 and the difference can only be explained by assuming an additional transport of albumin through the small pore system.

The identical  $C_p/C_t$  ratio of albumin and transferrin (molecular weight 70 000 and 80 000 respectively) is of interest considering the difference in the electrical charge of the two molecules (2.1 at pH 8.6, isoelectric points 4.9 and 5.9 respectively) and suggests that the permeability of vessels to proteins is a function of molecular size.

We are grateful to Mr Ove Forsberg for the skilful technical assistance in the electrophoretic analyses of this study.

## References

- ALLAN, A. and H. O. FARMER. Protein concentration of interstitial fluid collected from rat skin by microdialysis. *Acta physiol. scand.* 1973 88: 350-358.
- BROWN, J. A., K. S. KIM and J. L. BOLLMAN. Extravascular diffusion of proteins. *Amer. J. Physiol.* 1955, 187: 17-220.
- BRUNE, B. and G. PALADE. Studies on blood capillaries. *J. cell. Biol.* 1968, 37: 244-299.
- CASLEY-SMITH, J. R. The distribution and numbers of small vesicles in blood and lymphatic endothelium and in mesothelium. *J. Anat.* 1968, 103: 202-203. (Abstract)
- CASLEY-SMITH, J. R. The functioning and interrelationship of blood capillaries and lymphatics. *Experimental (Basel)* 1976, 32: 1-10.

- CARLEY SMITH, J. R. and T. BOLTON. The presence of large colloidal osmotic pressures across large pores. *Microvasc Res* 1973 5 213-216.
- CLIFF, W. J. and P. A. NICOLL. Structure and function of lymphatic vessels of the bat wing. *Quart J exp Physiol* 1970 55 112-111.
- COURTICE, F. C. Determination of protein fractions in small samples of plasma and of lymph in the rabbit. *Aust J exp Biol med Sci* 1960 38 393-402.
- DAVIS, B. J. Disc-electrophoresis. II. Method and application to human serum proteins. *Ann NY Acad Sci* 1964 121 404-426.
- GALLICK, D. G. and E. M. RENKIN. Transport of large molecules from plasma to interstitial fluid and lymph in dogs. *Amer J Physiol* 1970 219 1595-1605.
- GROTTI, G. Passage of dextran molecules across the blood lymph barrier. *Acta chir scand* 1956 Suppl 211 1-84.
- HAJAMAS, H. and H. FREDEN. Comparative analysis of the protein contents of local subcutaneous tissue fluid and plasma. *Microvasc Res* 1970 2 163-171.
- INTAGLIETTA, M. R. F. PAWULA and W. R. TOMKINS. Pressure measurements in the mammalian microvasculature. *Microvasc Res* 1970 16 294-300.
- KARNOWSKY, M. J. The ultrastructural basis of capillary exchange. *J gen Physiol* 1968 52 64-95.
- LARSEN, N. A. II H. PARYDO and M. ROSSINO. Filtration as the main mechanism of overall transcapillary protein escape from the plasma. *Microvasc Res* 1974 7 1-iv Editorial.
- LEAK, L. V. and J. F. BURKE. Ultrastructural studies on the lymphatic anchoring filaments. *J cell Biol* 1968 36 179-149.
- MICHEL, C. C. The transport of solutes by osmotic flow with particular reference to the transport of proteins across the clefts fenestras of lateral capillaries. *Microvasc Res* 1974 8 122-14.
- ORNSTEIN, L. Disc-electrophoresis. I. Background and theories. *Ann NY Acad Sci* 1964 121 321-346.
- PALADE, G. F. Fine structure of blood capillaries. *J appl Physiol* 1953 24 14-4 (Abstract).
- RENKIN, E. M. Filtration, diffusion, and molecular sieving through porous cellulose membrane. *J gen Physiol* 1954 38 225-243.
- RICHARDSON, D. R. and B. W. ZWEIFACH. Pressure relationship on the macro- and micro-circulation of the mesentery. *Microvasc Res* 1970 2 474-488.
- RUTILI, G. and K. E. ARFORS. Fluorescent labelled dextran measurement in interstitial fluid a studies on macromolecular permeability. *Microvasc Res* 1976. In press.
- SWEDS, S. Analysis of thyoid proteins by means of micro disc-electrophoresis. *J Chromatograph* 1968 44 148-160.
- STUDER, R. and J. POTCHEN. The radioisotopic assessment of regional microvascular permeability to macromolecules. *Microvasc Res* 1971 3 35-48.
- WALKER, W. G. R. S. ROSS and J. D. S. HAMMOND. Study of the relationship between plasma volume and transcapillary protein exchange using  $^{125}$ I labelled albumin and  $^{125}$ I-labelled globulins. *Circulat Res* 1960 8 1028-1040.
- WIEDERHJELM, C. A. Dynamics of capillary fluid exchange. Proc. Int. Union of Physiol. Sciences. XXV Int. Congress, Munich 1971 Vol III. p. 461-26.

# Pineal Serotonin Metabolism in Non-Innervated Perinatal Glands before and after Intraocular Maturation Supersensitivity of Adrenoceptors that have never been Innervated

By

MARIA BÄCKSTRÖM, LARS OLSON and ÅKE SEIGER

Received 23 March 1976

## Abstract

BÄCKSTRÖM, M., L. OLSON and Å. SEIGER. *Pineal serotonin metabolism in non-innervated perinatal glands before and after intraocular maturation. Supersensitivity of adrenoceptors that have never been innervated.* Acta physiol. scand. 1977 99 9-18.

Transplantations were made of fetal pineal glands (crown-rump length, CRL, 19-30 mm) or pineal glands from adult male rats to the anterior chamber of the eye of the rat. Studies were performed with regard to the importance of the age of the donor animal (and thereby the degree of maturation and innervation of the gland to be transplanted) for the possible development of desensitization supersensitivity. The transplants were cultured in medium containing  $^3\text{H}$ -serotonin. Increased production of  $^3\text{H}$ -N-acetylheteronin (NAcS) was used as the main criterion for  $\beta$ -adrenergic stimulation. 4 experimental groups were obtained by transplanting fetal or adult pineals to intact or sympathetically denervated eyes. In all 4 groups  $\beta_1$  stimulation (KW 2033  $10^{-6}$  M) increased  $^3\text{H}$ -NAcS formation. The response to  $\beta$ -stimulation was significantly higher in denervated fetal pineal transplants than in innervated fetal transplants and thus demonstrating  $\beta$ -receptor supersensitivity. It was concluded that: a) the ability to respond to  $\beta$ -adrenoceptor stimulation with increased  $^3\text{H}$ -NAcS formation develops between the 16th and 20th day of gestation, b) transplants derived from fetal as well as from adult rats can respond to  $\beta$ -adrenergic stimulation, c) this sensitivity also develops in acute transplants that at the time of transplantation lacked the capacity to increase their  $^3\text{H}$ -NAcS formation in response to treatment with  $\beta$ -agonist, d) desensitization supersensitivity occurs in fetal transplants that become mature and e) sympathetically denervated eyes.

**Key words:** Pineal, transplants, serotonin,  $\beta$ -adrenergic, desensitization supersensitivity, resensitization.

The rat pineal gland metabolizes serotonin to N-acetylheteronin (NAcS) and melatonin (MEL) or 5-hydroxyindoleacetic acid (5-HIAA) (Wurtman *et al.* 1968). The activity of N-acetyltransferase (NAT), N-acetylating serotonin to NAcS (Weissbach *et al.* 1960), is controlled by  $\beta$ -adrenergic stimulation of the pineal noradrenoceptor mediated through increased intracellular production of cyclic adenosine 3',5'-monophosphate (cAMP) (Klein, Berg and Weller 1970). In our previous study homologous intraocular transplants from newborn rats were assayed 2.5-3.0 months following transplantation and were found to have

matured and to have become innervated by sympathetic nerves. They were responsive to external lighting conditions with pronounced increases of NAT (20-1) and hydroxyindole-O-methyl transferase (HIOMT) activities during dark hours as compared to light activity (Bäckström Olson and Seiger 1976). Similar findings have recently been reported by Moore (1975). Sympathetic denervation of the host eye eliminated the dark induced rise in HIOMT activity and greatly attenuated NAT enzyme variation (2-1). Cholinergic iris nerves have been shown to readily reinnervate eye chamber transplants that receive cholinergic fibers in their normal locus (Takahara, Jacobowitz and Laties 1972). The constant presence of cholinergic nerves in all host irides did not modify the dependence on an intact adrenergic innervation.

The persistent low amplitude NAT variation in denervated eye chamber pineal transplants was statistically significant. One explanation for this variation could be the development of supersensitivity to circulating catecholamines in the transplants subsequent to denervation (Cannon and Rosenbluth 1949, Trendelenburg 1966). Development of denervation supersensitivity to  $\beta$ -adrenoceptor stimulation in rat pineal glands has been described (De Gucht and Axelrod 1972, 1973, Bäckström and Wetterberg 1973, Strada and Weiss 1974).

It was desired to investigate whether the degree of maturation and sympathetic innervation of the grafted pineals could influence the eventual status of indole metabolism. Would receptor supersensitivity in response to  $\beta$ -adrenoceptor stimulation develop in a pineal gland transplanted into a sympathetically denervated eye, at a stage prior to the sympathetic fiber development in the gland? Thus, would the receptor become supersensitive without ever having been contacted by its appropriate nerve supply?

### Materials and methods

**Animals.** Albino rats (Sprague-Dawley) were fed food pellets and water *ad libitum* and kept one (pregnant rats) or five to a cage. Lights were on between 06 and 18 h. Pregnant rats, used for direct organ culture of fetal pineals, were decapitated between 10 and 16 h. Uteri were quickly dissected out and kept on ice while preparations of tissues were performed. Pineal glands from fetal rats with CRL 20-21 mm, 50 mm and from 48 h old rats were each taken from one litter. Pineal glands from fetal rats with CRL 35 mm were taken from two litters. The fetal or newly born rats were decapitated and using a dissection microscope the pineal glands were freed from adherent tissue. Pineal glands were stored in ice cold medium for 1-2 min before being introduced into the culture chamber. Normal control rats were 300-350 g males, housed for 14 days on the same light-dark cycle. Host animals were sacrificed by decapitation between 9.30 and 12.30 h. The eyes were immediately removed and the transplants kept on ice during the dissection. Simultaneously processed *in situ* pineal glands were dissected out and at red in ice-cold incubation medium. All tissues were placed in the incubation chamber within approximately 3 min after decapitation.

**Transplantations.** Pineal glands from fetal rats with CRL 19-30 mm (18-20 days of gestation) and from adult male rats (150-200 g) were bilaterally transplanted into the anterior chamber of the eye, in adult recipient male rats. Pineals were rapidly dissected out from the donors, decapsulated and introduced into the anterior eye chamber through a slit in the cornea with a modified Pasteur pipette according to Olson and Malmfors (1970). All recipient animals (totally 33 rats) underwent unilateral sympathetic denervation by saturation of either the left or the right superior cervical ganglion a few days before transplantation. The cholinergic innervation of the recipient iris was in all cases left intact. The taking and development of the transplants were followed *in vivo* by repeated inspections through the cornea using a stereoscopic microscope under light ether anesthesia. The postoperative time ranged from 3 to 4 months.

**Organ culture.** Pineal glands from fetal rats and newly born rats were preincubated for 1-2 h before the  $\beta_1$ -adrenoceptor agonist KWD 2033 ( $10^{-8}$  M) (Carlsson *et al.* 1975) was added as indicated in Fig. 1. Pineal transplants and *in situ* pineal glands from experimental and normal rats were preincubated for 2-4

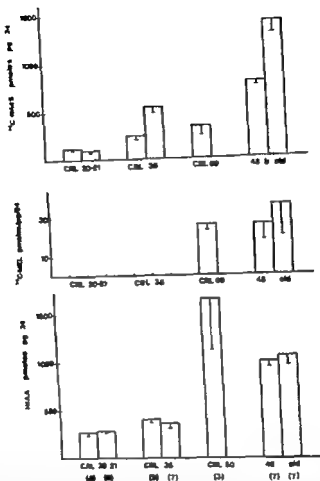


Fig. 1. Production of  $^{14}\text{C}$ -serotonin derivatives by cultured immature rat pineal glands.  $^{14}\text{C}$ -NAcS =  $^{14}\text{C}$ -Acetylserotonin,  $^{14}\text{C}$ -MEL =  $^{14}\text{C}$ -Melatonin,  $^{14}\text{C}$ -5-HIAA =  $^{14}\text{C}$ -5-Hydroxyindoleacetic acid. CRL values are given in parentheses in units of the animals used. Empty and shaded bars indicate mean values of  $^{14}\text{C}$ -labeled metabolite formation in unstimulated controls of KWD 2033 ( $10^{-6}$  M) stimulated glands respectively measured as picomoles of metabolite formed per pineal gland per 24 h culture period. Standard error of the mean is indicated by vertical lines on bars. The number of pineal glands cultured per group is presented above brackets below bars displaying  $^{14}\text{C}$ -5-HIAA formation. Shaded groups that differ significantly ( $p < 0.01$ ) from their respective control groups.

Following the preincubation period KWD 2033 ( $10^{-6}$  M) was added to some of the animals as stated in Table 1 and Fig. 2. All pineals were cultured according to the method developed by Trench (1959), modified by Raitz (1965) and first elaborated for pineal organ culture by Kline and Weller (1970). The glands were each cultured in 0.6 ml of BGJ<sub>1</sub>-medium, Finner-Jackson modification (Grand Island Biological Company). Bovine serum albumin (1 mg/ml), ascorbic acid (0.1 mg/ml) and 1-phenylethanol (0.3 mg/ml) were added on the day of the experiment. The medium contained 0.25 mM  $^{14}\text{C}$ -5-hydroxytryptamine (serotonin) creatine sulphate (Radiochemical Center, Amersham), specific activity 34 mCi/mmol, diluted with unlabeled serotonin (Fluka AG) to 4 mCi/mmol. No correction was made for the decrease in  $^{14}\text{C}$ -serotonin specific activity due to endogenous production of serotonin from 1-tryptophan contained in the culture medium. The culture chamber was continuously exposed to 21%  $\text{O}_2$  and 5%  $\text{CO}_2$  at 37°C. All tissues

TABLE 1 Production of  $^{14}\text{C}$  serotonin derivatives by cultured pineal glands and transplants.

Pineal types	Treatment		Metabolites in medium		
			N Acetyl 5-HT	Melatonin	5-HIAA
<i>In situ</i> normal	Control	(4)	152 $\pm$ 32	338 $\pm$ 67	3 930 $\pm$ 155
	KWD 2033	(7)	828 $\pm$ 146	656 $\pm$ 61	2 556 $\pm$ 473
<i>In situ</i> experimental	Control	(6)	167 $\pm$ 30	294 $\pm$ 45	3 120 $\pm$ 168
	KWD 2033	(4)	664 $\pm$ 215	688 $\pm$ 138	2 904 $\pm$ 528
All innervated transplants	Control	(16)	159 $\pm$ 21	170 $\pm$ 41	3 430 $\pm$ 344
	KWD 2033	(13)	512 $\pm$ 26	182 $\pm$ 41	3 078 $\pm$ 189
All denervated transplants	Control	(14)	186 $\pm$ 27	87 $\pm$ 22	3 406 $\pm$ 409
	KWD 2033	(16)	1 178 $\pm$ 96	332 $\pm$ 79	2 945 $\pm$ 377

Data are expressed as mean  $\pm$  standard error of the mean of picomoles  $^{14}\text{C}$  labelled metabolite formed/pineal per 24 h culture period, preceded by 1 h preincubation. N Acetyl-5-HT = N-Acetylserotonin; HIAA = 5-Hydroxyindoleacetic acid. Numbers in brackets designate the number of pineal glands or transplants cultured per group. KWD 2033 concentration was in all cases  $10^{-6}$  M. nd indicate groups that differ significantly in amounts of  $^{14}\text{C}$  metabolites formed as compared to their respective control group.  $p < 0.05$ ,  $p < 0.01$  and  $p < 0.001$  respectively.

cultured for 24 h following addition of  $\beta$ -adrenoreceptor agonist (Bäckström and Wetterberg 1973). AAs were collected and frozen at  $-20^\circ\text{C}$ . The  $^{14}\text{C}$ -labelled serotonin metabolites were separated by thin-layer chromatography as described by Klein and Nairdes (1969). Radioactivity was eluted in 95% ethanol. 0.1 M HCL Insta-Gel (Packard Instrument International S.A.) or Aquasol (New England Nuclear) were added and radioactivity determined by liquid scintillation (Packard Tri-Carb 3380, supplied with Absolute Activity Analyzer 544).

Doses of KWD 2033 were given as the final concentration of the base in culture medium.

Statistical analyses were performed by application of Student's two-tailed *t* test for comparison of means.

## Results

***In vivo* observations.** All pineals survived transplantation into the anterior chamber of the eye regardless of the stage of development at operation. They became rapidly vascularized from the iris of the host eye. The most immature pineals (CRL 19–21 mm) were approximately 1–1.1 mm at operation and grew *in oculo* to a final size of approximately 2–1.5 mm. Pineals from CRL stages 22–25 mm and 27–30 mm were initially somewhat larger and reached the same final size as the most immature pineals. Adult pineals were approximately 2–1.5 mm already at transplantation, and did not change size *in oculo*. Denervated transplants were visibly smaller than innervated transplants regardless of donor age.

***Immature pineals.*** Pineal glands from the most immature fetal rats (CRL 20–21 mm) formed  $^{14}\text{C}$  NAcs. The production increased with maturity (Fig. 1). After addition of KWD 2033 ( $10^{-6}$  M) the pineal glands from fetal rats with CRL 35 mm and 2 day old rats significantly increased their production of  $^{14}\text{C}$  NAcs (2.2 and 1.8 times respectively), compared to controls ( $p < 0.01$  and  $p < 0.001$  respectively).

There was no production of  $^{14}\text{C}$  MEL in pineal glands from fetal rats with CRL 20–21 mm or CRL 35 mm either untreated or following stimulation with KWD 2033 ( $10^{-6}$  M) (Fig. 1). Pineal glands from fetal rats with CRL 50 mm and from 2 day old rats produced minimal amounts of  $^{14}\text{C}$  MEL and this production was not altered by addition of drug.



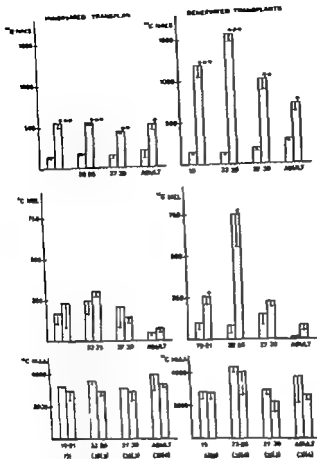


Fig. 2. Production of  $^{14}\text{C}$ -acetone derivatives by cultured transplanted and deservated rat pineal transplants derived from donor rats of various ages. The left part of the figure shows metabolite formation in transplanted transplants, the right shows deservated transplants.  $^{14}\text{C}$  NAC5 =  $^{14}\text{C}$ -N-Acetylserotonin,  $^{14}\text{C}$  MEL =  $^{14}\text{C}$  Melatonin,  $^{14}\text{C}$ -HIAA =  $^{14}\text{C}$ -5-Hydroxyindoleacetic acid. Numbers below bars give growth curve length (CRL) in mm of transplant donor animals. Empty and shaded bars illustrate mean values of  $^{14}\text{C}$ -labeled metabolite formation in untransplanted controls or KWD 2033 ( $10^{-6}$  M) stimulated transplants respectively measured as picomoles of metabolites formed per transplant per 24 h culture period. Standard error of the mean is indicated by vertical line in bar. The number of pineal transplants cultured per group presented (also brackets below the bars) each display  $^{14}\text{C}$ -5-HIAA formation. and significance groups that differ significantly from their respective control groups (p < 0.05, p < 0.01 and p < 0.001 respectively).

The production of  $^{14}\text{C}$ -5-HIAA also rose with age, reaching 25-30% of adult levels in 48 h old pineals (Fig. 1). KWD 2033 ( $10^{-6}$  M) did not affect the formation of  $^{14}\text{C}$ -5-HIAA.

**Normal mature and transplanted pineals.** *In situ* pineal glands from normal rats and experimental rats (carrying bilateral eye chamber transplants) produced amounts of  $^{14}\text{C}$ -NAC5,  $^{14}\text{C}$ -MEL and  $^{14}\text{C}$ -5-HIAA that did not differ statistically from each other when controls or KWD 2033 ( $10^{-6}$  M) treated groups were compared. In both types of glands KWD 2033 increased the production of  $^{14}\text{C}$ -NAC5 (5.4- and 4.0-fold respectively) and  $^{14}\text{C}$ -

MEL (1.9- and 2.3-fold respectively) ( $p < 0.05$ ) but did not alter  $^3\text{H}$ -5-HIAA formation (Table I).

When all non-stimulated transplants from the different donor groups were compared there was no difference between those that were sympathetically innervated and those that were denervated, in the formation of  $^3\text{H}$ -labelled NAcS, MEL or 5-HIAA per transplant (Table I).  $\beta$ -stimulation by KWD 2033 ( $10^{-6}$  M) caused a 2.7 times larger increase in  $^3\text{H}$ -NAcS production in denervated transplants than in innervated transplants ( $p < 0.001$ ). In the innervated group KWD 2033 increased  $^3\text{H}$ -NAcS formation 6.1 times (Table I). This large  $^3\text{H}$ -NAcS increase, induced by KWD 2033, was significantly higher than in the pineal glands of the experimental rats. KWD 2033 ( $10^{-6}$  M) also caused a 3.8-fold increase in the formation of  $^3\text{H}$ -MEL in denervated transplants ( $p < 0.01$ ). In innervated transplants there was no increase in  $^3\text{H}$ -MEL formation following KWD 2033 addition (Table I).

When innervated and denervated transplants were analyzed with respect to the nature of the donor animal it was found that the three different fetal groups were approximately equivalent (Fig. 2) while transplants obtained from adult donors were found to increase their  $^3\text{H}$ -NAcS formation less in response to  $\beta$ -stimulation. They also had lost much of their  $^3\text{H}$ -MEL synthesizing capacity.  $^3\text{H}$ -5-HIAA formation in transplants was not affected by denervation or drug treatment (Table I and Fig. 2).

### Discussion

In the denervated eye chamber pineal transplants a low amplitude variation of NAT enzyme activity persisted (Bäckström, Olson and Seiger 1976). This might have been due to development of denervation supersensitivity of the transplants, which might have responded to circulating catecholamines. The eye chamber transplantation technique also offered a possibility to study such a phenomenon in very immature fetal pineal glands that had not been innervated by the time of operation and where ganglionectomy would have posed technical difficulties. It may thus be possible that these fetal tissues develop an adrenergic receptor supersensitivity without ever having been in contact with their appropriate nerves.

Culture medium content of the 3  $^3\text{H}$ -serotonin metabolites NAcS, MEL and 5-HIAA (5-hydroxyindoleacetic acid) were assayed to obtain an estimate of NAT, HMOX and monoamine oxidase (MAO) enzyme activities respectively. Determination of  $^3\text{H}$ -5-HIAA was considered of importance to ascertain the specific adrenoceptor effect of the agonist and exclude a mere inhibition of serotonin decarboxylation. When this metabolic pathway which alternates with N-acetylation of serotonin, is inhibited a "mass-action" shunt over to N-acetylation has been suggested (Axelrod, Sheln and Wurtman 1969; Sheln 1971). A newly synthesized  $\beta_1$ -adrenoceptor agonist KWD 2033 (Carlsson *et al.* 1975) was chosen for the stimulation of pineal adrenoceptor mediated N-acetylation of  $^3\text{H}$ -serotonin. KWD 2033 has recently been shown to have maximal efficacy and potency for stimulation of rat pineal gland  $\beta$ -adrenoceptors in organ culture (Bäckström 1976). Metabolite production was measured in the culture medium, calculated per pineal, and no compensation was made for the differences in amount of tissue between *in situ* pineal glands and transplants.

**Ontogenetic development of serotonergic metabolism.** In earlier works pineals from fetal rats have been reported to lack responsive adenylyl cyclase which constitutes part of the proposed postjunctional adrenergic receptor (Wess 1971). However pineal glands from fetal rats with CRL 35 mm (about 20-21st day of gestation) are found to double their  $^3\text{H}$  NAcs formation in response to addition of the directly acting  $\beta$ -adrenoceptor agonist KWD 2033. This response was not observed in pineal glands from the most immature fetal rats with CRL 20-22 mm (about 17-18th day of gestation). NAT enzyme activity has been detected as early as 4 days before parturition. The initially high values of NAT decreased markedly around 7 days of age (Elfrson, Weller and Klein 1972). Similarly in comparison with mature pineal glands a higher unstimulated level of  $^3\text{H}$  NAcs was formed around and 2 days subsequent to birth as seen in our present cultures. Our findings are compatible with the report of Wess and Surada (1972) revealing a large increase of rat pineal phosphodiesterase activity between day 8 and 16 post partum. An increased breakdown of cAMP would lower the stimulation of postjunctional biochemical events required for maintaining NAT activity. HIOMT activity is hardly detectable during the first 10-12 days post partum in the rat pineal but then rises sharply to reach adult values at 34-50 days of age (Zweig and Snyder 1968, Snyder 1968, Klein and Lines 1969). A diurnal variation of HIOMT activity has first been detected in 39 day old rats (Klein and Lines 1969). Thus, the present absence or extremely low values of MEL production in very young rats were in good agreement with earlier reports. Low levels of MAO enzyme activity which is responsible for the deamination of serotonin to 5-HIAA, have been observed in pineals of newborn rats (Zweig and Snyder 1968, Snyder 1968). In the present organ cultures  $^3\text{H}$  5-HIAA formation was noted when pineals were explanted 4 days before birth, at a stage when pineals are not yet sympathetically innervated (Machado 1971). The  $^3\text{H}$  5-HIAA was thus formed extraneuronally probably also pinealocytes.

**Transplants.** Normal adult *in situ* pineal glands were cultured in the same experiment as pineal transplants and *in situ* pineal glands from experimental animals (carrying bilateral ocular transplants and submitted to unilateral superior cervical ganglionectomy). Pineal glands from normal adult rats did not differ in their production of  $^3\text{H}$ -labelled NAcs, MEL or 5-HIAA as compared to the *in situ* pineal glands from experimental rats either unstimulated or  $\beta$ -stimulated with KWD 2033. This shows that the one remaining superior cervical ganglion supplied the *in situ* pineal glands with sufficient sympathetic nerves to ensure a normal adrenergic response. It also follows that the eye chamber transplants did not influence the activity of the *in situ* pineal gland to any significant extent.

Denervated transplants from fetal donors gave a higher increase of  $^3\text{H}$ -NAcs production following  $\beta$ -stimulation with KWD 2033 than did innervated  $\beta$ -stimulated transplants taken from rats of corresponding ages indicating the development of supersensitivity to aminergic stimulation. This phenomenon was less pronounced in grafted pineals from adult donors although they were the only transplants which were actually denervated. Innervated grafts from all age groups responded to  $\beta$ -stimulation with an increased  $^3\text{H}$  NAcs production of about the same magnitude as normal pineal glands.  $^3\text{H}$  MEL formation in transplants was lower than in the *in situ* pineal glands both in denervated and innervated transplants. It was shown by Blakstrom, Olson and Seiger (1976) that denervation of transplants caused

an extinction of HIOMT diurnal variation. Thus, eye chamber transplants behaved similarly to *in situ* pineal glands in that they are also dependent on an intact sympathetic innervation for their diurnal HIOMT variation (Axelrod, Wurtman and Snyder 1965). From the present experiments it can be seen that 24 h incubation with a directly acting  $\beta$ -adrenoceptor agonist could increase the production of  $^{14}\text{C}$  MEL in denervated transplants but not in innervated transplants, although the latter displayed a dark induced rise in HIOMT activity *in oculo*. The reason for this discrepancy is not readily explained. Nagle, Cardinali and Rose (1973) found that intraperitoneal injections of noradrenaline caused significant increase in pineal HIOMT activity only in ganglionectomized rats and did not affect enzyme activity in intact animals. They interpreted these findings as a phenomenon of denervation supersensitivity. The present observations on  $^{14}\text{C}$  MEL formation in denervated transplants might be similarly explained. Pineals from adult donors grafted to sympathetically innervated eyes lose some of their capacity to form  $^{14}\text{C}$  MEL in spite of restoration of the sympathetic innervation. It is possible that the capacity of pineal grafts from adult donors to survive as functional eye chamber transplants is less than for fetal tissues.

Innervated and denervated transplants produced  $^{14}\text{C}$  5-HIAA in equally large amounts as the *in situ* pineal glands. Total MAO activity (measured in homogenates) has been reported to fall 30–50% following denervation of rat pineal glands (Snyder, Fischer and Axelrod 1965; Håkansson and Owman 1966; Snyder 1968) mainly through elimination of MAO-A isoenzyme activity (Johnston 1968) which is predominantly contained in the sympathetic nerve terminals in the rat pineal (Goridis and Neff 1971; Neff *et al.* 1974). Serotonin deamination was reported to decrease 70% as compared to normal pineals when measured in homogenates of chronically denervated pineal glands (Goridis and Neff 1971). Any denervation induced decrease in  $^{14}\text{C}$  5-HIAA formation was not revealed in the present experiment. This might be explained by a change of enzyme affinity for serotonin within the fraction of MAO enzyme remaining despite denervation. It can be noted that Håkansson and Owman (1965) found no decrease in pineal gland MAO activity for 21 days post-denervation when  $^{14}\text{C}$ -serotonin was used as substrate for incubations of intact glands. Bäckström and Wetterberg (1973) showed that chronically denervated *in situ* pineals cultured for 24 h in medium containing 0.25 mM  $^{14}\text{C}$  serotonin, produced as much  $^{14}\text{C}$  5-HIAA as did normal *in situ* pineal glands in culture.

We conclude that pineal glands transplanted into sympathetically innervated host eyes become innervated regardless of the state of innervation or lack of such at the time of operation. The present experiments lend support to the suggestion that denervated eye chamber pineal transplants might become supersensitive to aminergic stimulation and that this supersensitivity could explain the discreet NAT enzyme alternation between dark and light that was seen in denervated transplants. It is not necessary for a normally sympathetically innervated tissue ever to have been innervated to develop a "denervation" supersensitivity to sympathomimetic amines.

## References

1. J. H. M. SMITH and R. J. W. THOMAS, Suppression of  $C^{14}$ -melatonin synthesis from  $C^3$ -tryptophan by noradrenaline in rat pineal in organ culture. *Proc. nat. Acad. Sci. (Wash.)* 1969 62, 544-549.
2. J. H. M. SMITH and S. H. SVETKEY, Control of hydroxyindole-O-methyltransferase activity in rat pineal gland by environmental lighting. *J. Biol. Chem.* 1965, 240, 949-954.
3. J. H. M. SMITH, Effects of select  $\beta$ -adrenoceptor agonists on rat pineal gland serotonin metabolism. To be published.
4. J. H. M. SMITH, L. OLSAN and A. SEIGER, N-acetyltransferase and hydroxyindole-O-methyltransferase activity in intracocular pineal transplants. *Diurnal rhythm as evidence for functional sympathetic innervation. Acta physiol. scand.* 1976 96, 64-71.
5. J. H. M. SMITH and L. WETTERBERG, Increased N-acetylserotonin and melatonin formation induced by aporphine in rat pineal gland organ culture is  $\beta$ -adrenergic receptor mechanism. *Acta physiol. scand.* 1973 87, 11-120.
6. W. H. II and A. ROSENBLUTH, The superneutrality of dimerized structures. Macmillan, New York, 1949.
7. W. H. II, P. J. FRAMON, G. S. LEBOWITZ, H. SCHAEFER and A. WETTERBERG, *Deutsche Offenzugschrift* 19, 501-751.
8. J. H. M. SMITH and J. A. M. SMITH, Induction and superinduction of serotonin N-acetyltransferase by adrenergic agonists and denervation in rat pineal organ. *Proc. nat. Acad. Sci. (Wash.)* 1972 69, 2208-2211.
9. J. H. M. SMITH and J. A. M. SMITH, Superinduction of serotonin N-acetyltransferase and superneutrality of cryptochrome in denervated pineal gland. *Mol. Pharmacol.* 1973 9, 612-618.
10. W. H. II, J. L. W. LEE and D. C. KATZ, Development of circadian rhythm in the activity of pineal serotonin N-acetyltransferase. *J. Neurochem.* 1977 29, 1335-1341.
11. W. H. II and N. H. NAI, Evidence for a specific monoamine oxidase associated with sympathetic nerves. *Neuropharmacol.* 1971 10, 357-364.
12. W. H. II and C. OLSAN, Effect of denervation and enzyme inhibition on DOPA decarboxylase and monoamine oxidase activities of rat pineal gland. *J. Neurosci.* 1965 12, 417-429.
13. W. H. II and C. OLSAN, Pineal dopa decarboxylase and monoamine oxidase activities as related to the monoamine stores. *J. Neurochem.* 1966 13, 597-605.
14. W. H. II, J. P. Some observations upon a new inhibitor of monoamine oxidase in brain. *Neurochem. Res.* 1968 17, 1285-1297.
15. W. H. II, D. C. KATZ and J. W. LEE, Melatonin synthesis. Adenosine 3',5' monophosphate and noradrenaline stimulate N-acetyltransferase. *Science* 1970, 163, 979-980.
16. W. H. II and S. V. LINDA, Pineal hydroxyindole-O-methyltransferase activity in the growing rat. *Endocrinology* 1969 84, 1521-1523.
17. W. H. II and A. NOBES, Thin-layer chromatographic separation of pineal gland derivatives of serotonin. *C. Anal. Biochem.* 1969 31, 440-443.
18. W. H. II and J. W. LEE, Input and output signals in a model neural system. The regulation of melatonin production in the pineal gland. *Life* 1970, 6, 197-204.
19. W. H. II, A. B. H. Electron microscopy of developing sympathetic fibres in the rat pineal body. The formation of granular vesicles. In: *Progress in Brain Res.* (Ed. H. Eskin), 1971 34, 171-185.
20. J. R. Y. Pineal transplant to the anterior chamber of the eye: Evidence for functional reinnervation. *Eur. J. Neurosci.* 1975 49, 617-621.
21. W. H. II, C. A. D. P. CARDINALI and J. H. ROBERTS, Retinal and pineal hydroxyindole-O-methyltransferase in the rat. Changes following cervical sympathectomy, sympathectomy or bleeding. *Endocrinol.* 1973 1550-1564.
22. J. H. II, H. Y. T. Y. W., C. GORDON and H. BLAUER, The metabolism of indolealkylamines by type A and B monoamine oxidase of brain. I. *Advances in Biochem. Psychopharmacol. Raven Press, New York* 19, 11-31-58.
23. W. H. II and T. MAJUMDER, Growth characteristics of adrenergic nerves in the adult rat. Fluorescence histochemical and  $^3H$  noradrenaline uptake studies using tissue transplantation to the anterior chamber of the eye. *Acta physiol. scand.* 1970, Suppl. 348, 1-112.
24. W. H. II, Bone resorption in tissue culture. Factors influencing the response to parathyroid hormone. *J. Cell Biol.* 1965, 44, 123-116.
25. W. H. II, Control of melatonin synthesis by noradrenaline in rat pineal organ cultures. In: Wolsten-

an extinction of HIOMT diurnal variation. Thus, eye chamber transplants behaved similarly to *in situ* pineal glands in that they are also dependent on an intact sympathetic innervation for their diurnal HIOMT variation (Axelrod, Wurtman and Snyder 1965). From the present experiments it can be seen that 24 h incubation with a directly acting  $\beta$ -adrenoceptor agonist could increase the production of C MEL in denervated transplants but not in innervated transplants, although the latter displayed a dark induced rise in HIOMT activity *in oculo*. The reason for this discrepancy is not readily explained. Nagle, Cardinali and Rouse (1973) found that intraperitoneal injections of noradrenaline caused significant increase in pineal HIOMT activity only in ganglionectomized rats and did not affect enzyme activity in intact animals. They interpreted these findings as a phenomenon of denervation supersensitivity. The present observations on C MEL formation in denervated transplants might be similarly explained. Pineals from adult donors grafted to sympathetically innervated eyes lose some of their capacity to form C MEL in spite of restoration of the sympathetic innervation. It is possible that the capacity of pineal grafts from adult donors to survive as functional eye chamber transplants is less than for fetal tissues.

Innervated and denervated transplants produced  $^3\text{H}$ -5-HIAA in equally large amounts as the *in situ* pineal glands. Total MAO activity (measured in homogenates) has been reported to fall 30–50% following denervation of rat pineal glands (Snyder, Fischer and Axelrod 1965; Håkansson and Owman 1966; Snyder 1968) mainly through elimination of MAO-A-isoenzyme activity (Johnston 1968) which is predominantly contained in the sympathetic nerve terminals in the rat pineal (Gorklis and Neff 1971; Neff *et al.* 1974). Serotonin deamination was reported to decrease 70% as compared to normal pineals when measured in homogenates of chronically denervated pineal glands (Gorklis and Neff 1971). Any denervation induced decrease in C 5-HIAA formation was not revealed in the present experiment. This might be explained by a change of enzyme affinity for serotonin within the fraction of MAO enzyme remaining despite denervation. It can be noted that Håkansson and Owman (1965) found no decrease in pineal gland MAO activity for 21 days post denervation when  $^3\text{H}$ -serotonin was used as substrate for incubations of intact glands. Bäckström and Wetterberg (1973) showed that chronically denervated *in situ* pineals cultured for 24 h in medium containing 0.25 mM  $^3\text{H}$ -serotonin, produced as much  $^3\text{H}$ -5-HIAA as did normal *in situ* pineal glands in culture.

We conclude that pineal glands transplanted into sympathetically innervated host eyes become innervated regardless of the state of innervation or lack of such at the time of operation. The present experiments lend support to the suggestion that denervated eye chamber pineal transplants might become supersensitive to aminergic stimulation and that this supersensitivity could explain the discreet NAT enzyme alternation between dark and light that was seen in denervated transplants. It is not necessary for a normally sympathetically innervated tissue ever to have been innervated to develop a "denervation" supersensitivity to sympathomimetic amines.



- holme, O. E. W. and J. Knight (eds) *The Pineal gland*. Ciba Foundat. Sympos. Churchill Livingstone, Edinburgh and London, 1971, 197-207.
- SNYDER, S. H., J. FISCHER and J. AXELROD, Evidence for the presence of monoamine oxidase in sympathetic nerve endings. *Biochem. Pharmacol.* 1965, 14, 363-365.
- SNYDER, S. H., Development of enzyme activities and a circadian rhythm in pineal gland serotonin. Evidence for a nonretinal pathway of light to the pineal gland of newborn rats. *Advanc. Pharmacol.* 1972, Part A, 6, 301-305.
- STRADA, S. J. and B. WEISS, Increased response to catecholamines of the cyclic AMP system of rat pineal gland induced by decreased sympathetic activity. *Arch. Biochem.* 1974, 160, 197-204.
- TRENDELENBURG, U., Mechanisms of supersensitivity and subsensitivity to sympathomimetic amines. *Pharmacol. Rev.* 1966, 18, 629-640.
- TROWELL, E. A., The culture of mature organs in a synthetic medium. *Exp. B. Res.* 1959, 16, 118-141.
- TSUKAHARA, S., D. JACOBOWITZ and A. M. LATIES, Cholinergic reinnervation of iris transplants to the anterior chamber of the eye. *Amer. J. Ophthalmol.* 1972, 73, 394-399.
- WEISS, B., Ontogenetic development of adenylyl cyclase and phosphodiesterase in rat brain. *J. Neurochem.* 1971, 18, 469-477.
- WEISS, B. and S. J. STRADA, Neuroendocrine control of the cyclic AMP system of brain and pineal gland. *Advanc. Cyclic Nucleotide Res.* 1972, 1, 357-374.
- WEISSBACH, H., B. G. REDFIELD and J. AXELROD, Biosynthesis of melatonin. Enzymatic conversion of serotonin to N-acetylserotonin. *Biochem. biophys. Acta (Amst.)* 1960, 43, 352-353.
- WURTMAN, R., J. F. LARIN, J. AXELROD, H. M. SHEN and K. ROSASCO, Formation of melatonin and 5-hydroxyindole acetic acid from  $^{14}\text{C}$  tryptophan by rat pineal glands in organ culture. *Nature (Lond.)* 1968, 217, 953-954.
- ZWED, M. H. and S. H. SNYDER, The development of serotonin and serotonin-related enzymes in the pineal gland of the rat. *Communic. Behav. Biol.* 1968, Part A, 1, 103-108.



## Local Reflex in Microcirculation in Human Skeletal Muscle

By

O. HENRIKSEN and P. SEJRSØN

Received 26 April 1976

---

### Abstract

---

HENRIKSEN, O. and P. SEJRSØN. *Local reflex in microcirculation in human skeletal muscle.* Acta physiol. scand. 1977 99 19-26.

The effect of venous stasis of 40 mmHg upon blood flow in human skeletal muscle was studied in four normal subjects and in six chronically sympathectomized patients. Blood flow in skeletal muscle was measured by the local  $^{133}\text{Xe}$  isotope technique. Blood flow decreased about 30 per cent during venous stasis of 40 mmHg. In "pseudo-vascular bed" induced by means of histamine, blood flow decreased only by 16 per cent, indicating that the decrease in blood flow is due to vasoconstrictor responses to increase in vascular transmural pressure. The vasoconstrictor response was unaffected by spinal sympathetic blockade, but was blocked in areas infiltrated with lidocaine or with phenolase. The vasoconstrictor response was present in the nonoperated limbs used as control, but abolished in the denervated areas in the two chronically sympathectomized patients. The findings strongly suggest that the vasoconstrictor response in skeletal muscle is due to local nervous mechanisms involving adrenergic fibres. Thus, local reflex mechanism, most likely sympathetic reflex, seems to be present in human skeletal muscle in its cutaneous and subcutaneous tissue. This indicates that about 45 per cent of the change in total vascular conductance, when person changes from supine to upright position, is due to this local reflex mechanism operating independently of the central nervous system.

---

Blood flow in human skeletal muscle decreased about 30 per cent when the leg was placed in dependent position (Balldin *et al.* 1971, Amery *et al.* 1973). This vasoconstrictor response to increase in vascular transmural pressure has also been demonstrated in human subcutaneous adipose tissue (Henriksen, Levin Nielsen and Paaske 1973) and human cutaneous tissue (Henriksen *et al.* 1973). The vasoconstrictor response has been shown to be due to local sympathetic reflex mechanism in these tissues (Henriksen and Abner 1975, Henriksen 1976 a, b, c, and d, Henriksen and Sejrsen 1976 a).

The purpose of the present study was to investigate whether there is a local reflex mechanism in the microcirculation in human skeletal muscle also. If so, the local reflex mechanism might account for a great deal of the total change in vascular conductance during changes from supine to upright position.

## Methods

The experiments were carried out in four healthy subjects and two chronically sympathectomized patients operated because of manual hyperhidrosis.

Room temperature was about 22°C and remained constant during the investigations. Blood flow in the anterior tibial muscle was estimated by the local  $^{133}\text{Xe}$  washout technique (Lassen, Lindbjerg and Mølgaard 1964). The  $\gamma$ -emission was detected by means of NaI(Tl) scintillation detectors, collimated to record only from the distal half of the labelled area in order to avoid interference from activity emitted by  $^{133}\text{Xe}$  accumulating in fat tissue along the veins. The measurements were started 20 min prior to order to minimize the interference of the injection trauma. Increase in arterial transmural pressure was induced by occlusion. A cuff was placed on the thigh at the upper rim, and the  $^{133}\text{Xe}$  washout at constant  $k$  was measured consecutively 1) with the cuff deflated,  $k_{\text{unperf}}$ ; 2) with the cuff inflated to 40 mmHg,  $k_{\text{perf}}$ ; and 3) with the cuff deflated,  $k_{\text{ref}}$ . Each period of measurement lasted about 1 min, and the count rate was printed out every 10 or 20 s.

### I. Animals

(a) *Cervical nervous blockade* In order to test the possible contribution by central reflex mechanisms the effect of spinal sympathetic blockade upon the response to occlusion was investigated in one subject. A catheter was placed in the epidural space before the measurements were started. The experiment commenced before and 3 after the epidural anesthesia had been induced by marcaine (Henriksen and Alsner 1975). The sweat test (Djunerjatz 1960) was performed before and after the infusion of marcaine, and after the measurements were terminated.

(b) *Local nervous blockade* The effect of local nervous blockade on lipofluorescent block of upon the response to occlusion was studied by injecting 1 ml of phenolamine (10 mg/ml) and 1 ml of 1% novocaine without vasoconstrictant (20 mg/ml, 3:10 mol/l) mixed with 0.2 ml  $^{133}\text{Xe}$  isotonic saline into the anterior tibial muscle. The experiments were started 70 min later with the subject placed in a supine position.

(c) *Blockade of the anterior smooth muscle* A peripheral arterial bed was induced by injecting 0.1 ml of 1% starting as the base (1 mg/ml) mixed with  $^{133}\text{Xe}$  into the anterior tibial muscle. The activity was recorded every 5 s, and each period of measurement lasted only 15 min.

(d) *Measurement of venous pressure* Venous pressure was measured directly in the dorsum of the hand during occlusion. One subject placed in a supine position.

### II. Chronic sympathetic denervation

(a) The experiments were performed on the brachioradial muscle in both arms of a unilaterally sympathectomized patient, operated about 9 years before. The hand on the side of operation was completely dry. There was still manual hyperhidrosis on the other side (control side). The effect of occlusion upon muscle blood flow was studied in both arms.

(b) In a bilaterally sympathectomized patient, studied 1 year postoperatively, measurements were performed on the brachioradial muscle, and the anterior tibial muscle was used as control.

### Calculations and statistics

Mean perfusion coefficient,  $f$ , is calculated from the Kety equation  $f = k \lambda / 100$  (ml/100 g min) (Kety 1951), where  $k$  denotes the washout rate constant in min<sup>-1</sup> and  $\lambda$  the muscle tissue blood partition coefficient (ml/g) (0.7 ml/g) (Lassen *et al.* 1964).

Relative blood flow  $f_{\text{rel}}$  equals relative  $^{133}\text{Xe}$  washout rate constant,  $k_{\text{rel}} = k_{\text{perf}}/k_{\text{unperf}}$ . The tissue blood partition coefficient,  $\lambda$ , is assumed to be equal in the two situations.  $k_{\text{ref}}$  and  $f_{\text{ref}}$  here denote a reference value of the washout rate constants (min<sup>-1</sup>) and the perfusion coefficients (ml/100 g min) obtained just before and after the test respectively.

$k_{\text{unperf}}$  and  $f_{\text{ref}}$  were compared by means of the Student's *t*-test for paired samples. The calculated relative blood flow values obtained during tests and under the different conditions were compared by means of the randomization test for unpaired samples. Level of significance was chosen 0.05.

## Results

Blood flow decreased 30 per cent during occlusion of 40 mmHg in all 4 subjects (Figs. 1 and 2).

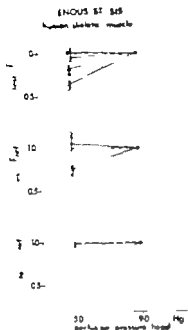


Fig. 1



Fig. 2

Fig. 1. Relative blood flow in human skeletal muscle during venous stasis of 40 mmHg, obtained from normal subjects plotted against perfusion pressure head (mmHg).  $I_{\text{rel}}$  denotes perfusion coefficient obtained during venous stasis, and  $I_{\text{rel}0}$  the stage perfusion coefficient calculated from the stasis obtained just before and after the test: control,  $\bullet$  areas infiltrated with phenolamine,  $\text{—}$  areas infiltrated with histamine. Areas infiltrated with histamine. Vertical lines with bars denote  $\pm$  S.E. Figures denote number of cases.

Fig. 2. Relative blood flow in human skeletal muscle during venous stasis of 40 mmHg, obtained before and after spinal sympathectomy blockade induced by epidural anesthetic, plotted against perfusion pressure head before blockade,  $\bullet$  after blockade. Vertical lines with bars denote  $\pm$  S.E. Figures denote number of cases.

The cut test became negative after the epidural anesthesia had been induced and was still negative after conclusion of the measurements, indicating that the sympathetic fibres had been blocked. Arterial blood pressure remained almost constant during the measuring period.  $I_{\text{rel}}$  obtained just after the blockade was effective was approximately 50 per cent higher than that obtained just before the blockade. Blood flow decreased 40% before ( $p < 0.01$ ) and 35% ( $p < 0.05$ ) after the blockade during venous stasis of 40 mmHg (Fig. 2). There was no difference in the response ( $p > 0.7$ ).

Lidocaine and phenolamine blocked the vasoconstrictor response to venous stasis as blood flow remained constant corresponding to decrease in vascular resistance (Fig. 1). When lidocaine (3–10 mol/l) was applied, relative blood flow  $\pm$  S.E. was  $0.97 \pm 0.05$  ( $p > 0.6$ ).

When the tissue was infiltrated with histamine,  $I_{\text{rel}}$  increased from  $0.017 \text{ min}^{-1}$  to  $0.56 \text{ min}^{-1}$ . During venous stasis, blood flow in these areas decreased 16 per cent ( $p < 0.001$ ) (Fig. 1). This response differed significantly from that obtained in controls ( $p < 0.001$ ).

vascular smooth muscle cells, affecting myogenic reactivity. Lidocaine ( $3 \cdot 10^{-4}$  mol/l) did not affect myogenic activity in the rat portal vein, but blocked impulse transmission in sympathetic fibres effectively (Johansson and Ljung 1967). In this concentration lidocaine blocked the vasoconstrictor response effectively. In two experiments phentolamine,  $5 \cdot 10^{-4}$  mol/l was applied. Even in this low concentration phentolamine blocked the vasoconstrictor response to venous stasis induced 15 min after the injection. Four control expts. with injection of isotonic saline performed in order to test the effect of the injection trauma indicated that this trauma was without effect 15 min after the injection. Phentolamine ( $5 \cdot 10^{-4}$  mol/l) did not affect myogenic activity in the rat portal vein (Johansson and Mellander 1973).

The effect of lidocaine and phentolamine is, therefore, most likely due to a blockade of sympathetic adrenergic vasoconstrictor fibres, indicating that the vasoconstrictor response is due to a local nervous mechanism.

Folkow and Öberg (1961) observed that venous stasis of 10 mmHg induced a decrease in blood flow in skeletal muscle in reserpinized cats, indicating that vascular reactivity is still present in more or less complete catecholamine depleted tissue. The duration of the periods of measurement was less than 40 s. Jones and Berne (1964) observed a transient decrease in blood flow in the thigh muscle of dog during venous stasis of 70 mmHg, lasting about 40 s, whereafter blood flow almost returned to the level before the period of stasis. Lundvall and Mellander (1976) made similar observations in skeletal muscle of cat during external negative pressure of 40 mmHg. These observations suggest an intrinsic vascular response to increase in vascular transmural pressure.

In humans a steady decrease in blood flow was observed during the venous stasis. 5 days after sympathectomy this response was abolished, indicating that the vasoconstrictor response to venous stasis in humans rather is neurogenic than an intrinsic vascular response.

The findings in the reserpinized cats (Folkow and Öberg 1961) is in agreement with the observation that autoregulation in subcutaneous tissue was retained years after the sympathectomy (Henriksen 1976 b). This observation does not exclude an overlying nervous reinforcing mechanism.

When increase in venous transmural pressure during lowering of the limb by 40 cm was prevented by letting the subject tip his foot the vasoconstrictor response was blocked in subcutaneous tissue as vascular resistance increased only by 48% corresponding to the increase in arterial perfusion pressure head (autoregulation) (Henriksen and Sejrsen 1976 b). The abolishment of the vasoconstrictor response during exercise might be due to liberation of metabolites in subcutaneous tissue affecting myogenic activity. However elevation of venous transmural pressure during exercise by inducing venous stasis elicited an increase in vascular resistance of the same magnitude as in the lowered, resting leg. In patients with venous insufficiency exercise did not prevent the increase in venous transmural pressure and did not abolish the vasoconstrictor response. Thus the abolishment of the vasoconstrictor response to lowering during exercise, is probably due to prevention of increase in venous transmural pressure, indicating that the vasoconstrictor response depends upon an impulse transmission from veins to arterioles. This furthermore strongly suggests that the vasoconstrictor response is due to a local nervous mechanism, probably a sympathetic axon reflex.

The findings in the present study are similar to those obtained in human cutaneous tissue and human subcutaneous adipose tissue (Henriksen and Sejrsen 1976 a, Henriksen 1975, Henriksen 1976 a, b, c, d). This means that blood flow in cutaneous tissue, subcutaneous adipose tissue, and skeletal muscle decreases about 50% due to this local reflex mechanism, when venous transmural pressure is elevated about 25 mmHg or more.

### Plethysmography

Blood flow measured by venous plethysmography might be influenced by the reflex. With a cuff pressure of 40 mmHg it took between 40 and 60 s for the venous pressure to increase to the threshold level of the reflex, 25 mmHg, indicating that the reflex is not elicited during venous occlusion plethysmography for measurements of blood flow at low venous pressures.

When the method is used for measurement of capillary filtration capacity (Mellander and Öberg and Odellström 1964), a possible involvement of the reflex should be considered.

### Homeodynamic implications of the reflex

1) Postural changes from supine to upright position caused an average decrease in cardiac output of 17 l/min (Hansson, Tabaken and Levy 1968), corresponding to a decrease in total vascular conductance of about 22%. Assuming that venous transmural pressure increases more than 25 mmHg in tissues corresponding to 50% of the b.w.t. and that the average decrease in blood flow is 2 ml/100 g/min in these tissues, the local reflex mechanism would account for a decrease in vascular conductance of about 10%, which is 45% of the total change. This means that about 45% of the postural changes in vascular tonus is due to the local reflex, occurring independently of the central nervous system. The remaining 55% are probably due to central reflex mechanisms elicited from baroreceptors located in the large arteries, carotid sinus, and heart (Roddie and Shepherd 1957, Beiser *et al.* 1970, Rowell 1973 and Brongelmann 1973).

2) Autoregulation of blood flow (i.e. maintenance of constant blood flow during changes in arterial perfusion pressure head) became evident when the reflex was blocked, indicating that autoregulation is due to intrinsic mechanisms.

### References

- ALNER, A. H., BRUNAR, M., DERLITTE, L., VANDERLINDER, L. and VERSTRAAT, J. Influence of body position on leg blood flow. *Scand. J. Clin. Lab. Invest.* 1973 31 Suppl. 128, 29.
- BAL, O., L. C. E. E. LINDGREN, J. LINDGREN, and S. MELLANDER. Changes in distribution of  $^{133}\text{Xe}$  from the anterior tibial muscle in man induced by isometric exercise and by shift in body position. *Arterioscler. Med.* 1971 1: 489-493.
- BAL, O., D. R. ZILMER, S. E. LIPSTON, D. T. MASON and E. BRUNAR WARD. The Role of Skin and Muscle Venous Vessels in Reflexes Mediated by the Baroreceptor System. *J. Clin. Invest.* 1970 49: 225-231.
- CHAMBERLAIN, A. B. F., CHAMBERLAIN, J. and S. M. LINDGREN. Nervous and local chemical control of pre-capillary sphincters in skeletal muscle as measured by changes in filtration coefficient. *Acta physiol. Scand.* 1965 5: 180-197.
- CHAMBERLAIN, A. B. F. Liquidation distribution of radiocesium in human venous-hemoglobin association curve. *J. Appl. Physiol.* 1966 16: 1065-1070.
- CHAMBERLAIN, A. B. F. and A. WILSON. A. Hemodynamic Test — an objective method for testing local anesthetic drugs. *Acta anaesth. Scand.* 1960 4: 189-198.

- FOLLOWER, B. and B. ÖBERG. Autoregulation and basal tone in consecutive vascular sections of skeletal muscles in reserpine-treated cats. *Acta physiol. scand.* 1961 53 105-113.
- HANFON, J. S., B. S. TALAMON and A. M. LEVY. Comparative exercise-cardio-respiratory performance of normal men in the third, fourth, and fifth decades of life. *Circulation* 1968. 37 345-360.
- HENRIKSEN, O. Effect of somesthetic denervation upon the vasoconstrictor response in human subcutaneous tissue. *Acta physiol. scand.* 1976 a. 96, 431-432.
- HENRIKSEN, O. Effect of chronic sympathetic denervation upon blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1976 b. 97 377-384.
- HENRIKSEN, O. Local nervous mechanism in regulation of blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1976 c. 97 385-391.
- HENRIKSEN, O. Local reflex in microcirculation in human subcutaneous tissue. *Acta physiol. scand.* 1976 d. 97 447-456.
- HENRIKSEN, O. and T. ALSTER. Effect of spinal sympathetic blockade upon local regulation of blood flow in subcutaneous tissue. *Acta physiol. scand.* 1975 95 83-88.
- HENRIKSEN, O. and P. SEJRSØ. Local reflex in microcirculation in human cutaneous tissue. *Acta physiol. scand.* 1976 a. 98 227-231.
- HENRIKSEN, O. and P. SEJRSØ. Effect of "veinpump" activation upon venous pressure and blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1976 b. Suppl. 440. Abstract No. 197 p. 129.
- HENRIKSEN, O., S. L. NIELSEN, W. P. PAASKE and P. SEJRSØ. Autoregulation of blood flow in human subcutaneous tissue. *Acta physiol. scand.* 1973 89 538-543.
- JOHANSSON, B. and B. LUND. Spread of excitation in the smooth muscle (III) the rat portal vein. *Acta physiol. scand.* 1967 70 312-322.
- JOHANSSON, B., B. LUND, T. MALMÖR and L. OLSSON. Denervation supersensitivity in the innervated smooth muscle of the portal vein. *Acta physiol. scand.* 1969 Suppl. 330 58.
- JOHANSSON, B. and S. MELLANDER. Static and dynamic components in vascular myogenic response: changes in length as revealed by electrical and mechanical recordings from the rat portal vein. *Circ. Res.* 1975. 36 76-83.
- JONES, R. D. and R. M. BERRI. Local regulation of blood flow in skeletal muscle. *Circulation* 1975 Suppl. 1 30-38.
- LARSEN, N. A., L. F. LINDBERG and O. MUNCK. Measurement of blood flow through skeletal muscle by intramuscular injection of Xenon-133. *Lancet* 1964 i 686.
- LUNDVALL, J. and S. MELLANDER. 1976. Personal communication.
- MELLANDER, S., B. ÖBERG and H. ODELMAN. Vascular adjustments to increased transmural pressure in cat and man with special reference to shifts in capillary fluid transfer. *Acta physiol. scand.* 1964. 6
- ROODIE, J. C. and J. T. SHEPHERD. The effects of carotid artery compression in man with reference to changes in vascular resistance in the limbs. *J. Physiol. (Lond.)* 1957 139 377-384.
- RUTWILL, L. B., C. R. WYSS and G. L. BRUNGELMANN. Sustained human skeletal muscle vasodilation with reduced baroreceptor activity. *J. appl. Physiol.* 1973. 35 639-643.
- SEJRSØ, P. and K. H. TUNNSEN. Shunting by diffusion of inert gas in skeletal muscle. *Acta physiol. scand.* 1972. 86. 82-91.
- TUNNSEN, K. H. and P. SEJRSØ. Inert gas diffusion method for measurement of blood flow. Comparison of bolus injection to directly measured blood flow in isolated gastrocnemius muscle. *Circulation* 1967 20, 552-564.
- TUNNSEN, K. H. and P. SEJRSØ. Washout of  $^{133}\text{Xe}$  after intramuscular injection and direct measurement of blood flow in skeletal muscle. *Scand. J. Clin. Lab. Invest.* 1970 25 71-81.

## **Efflux of 5-Hydroxytryptamine from Synaptosomes of Rat Cerebral Cortex**

By

SVANTE B. ROSS and DIANA KJELDER

Received 30 April 1976

### **Abstract**

ROSS, S. B. and D. KJELDER. *Efflux of 5-hydroxytryptamine from synaptosomes of rat cerebral cortex*. Acta physiol scand. 1977 99 27-36.

The efflux of  $^3\text{H}$ -5-HT from crude synaptosome preparation of the cerebral cortex of reserpinized rats is examined. The synaptosomes are loaded with  $^3\text{H}$ -5-HT by pre-incubation of the homogenate in presence of pargoline and desipramine in order to inhibit degradation of 5-HT and uptake into noradrenergic neurons. The synaptosomes were collected by centrifugation, washed and resuspended in 0.25 M sucrose. The spontaneous efflux of 5-HT was detectable at 0°C but marked efflux was observed at 27°C and 37°C. 4-Chloroamphetamine, low external Na<sup>+</sup> concentration, ouabain and the depolarizing agent veratrine markedly accelerated the initial (3 min) efflux. Inhibitors of the neuronal 5-HT uptake, e.g. chlorispropamine, II 102 09 and A 23189 antagonized the 5-HT efflux evoked by these means, whereas desipramine, which is a poor inhibitor of the 5-HT uptake, had only slight effect on the 5-HT efflux. It is suggested that 5-HT can be actively transported out from the synaptosomes by the reversed 5-HT uptake mechanism.

**Key words:** 5-hydroxytryptamine efflux, 4-chloroamphetamine, chlorispropamine, II 102 09, A 23189

The physiological importance of the neuronal membranal uptake mechanisms for the biogenic monoamines is generally recognized and accepted. Due to the hydrophobic nature of these amines they slowly pass the neurone membranes by diffusion and active uptake mechanisms are therefore necessary for a rapid transport of these amines through the membranes. It is also a general accepted view that the indirectly acting sympathomimetic amines exert their action by releasing the biogenic monoamines from intraneuronal binding sites (Trendelenburg 1973). From pharmacological observations it has been concluded that this release is different from that produced by nerve stimulation (Furchgott *et al.* 1963). Recently Thoa *et al.* (1975) found that the release of noradrenaline by the sympathomimetic amines in contrast to that of nerve stimulation in the isolated vas deferens was not joined by release of the enzyme dopamine- $\beta$ -hydroxylase. If the release of the biogenic amines does not occur with an exocytosis mechanism the problem remains how the released amines pass the neurone membranes outwardly since the same barrier exists in both directions. Until recently this problem has been overlooked, although it was suggested as possible that

TABLE I Inhibition of the efflux of H 5-HT from synaptosomes evoked by 4-chloroamphetamine ( $5 \times 10^{-6}$  M), low external Na ( $\sim 6$  mM NaCl replaced by 250 mM sucrose) and by veratridine ( $5 \times 10^{-6}$  M). The cortical synaptosomes were loaded with H 5-HT ( $1 \times 10^{-6}$  M) in presence of  $4 \times 10^{-4}$  M pargiline and  $3 \times 10^{-6}$  M desipramine for 20 min. The efflux of H 5-HT was determined incubating 100  $\mu$ l of the washed suspension in  $\sim 0$  ml Krebs buffer or modified buffer for 5 s at 37°C. The difference in the amount of radioactivity in the pellet in absence and presence of the efflux inducer was taken as the evoked efflux. The inhibition of the evoked efflux was calculated in per cent. Each value is the mean  $\pm$  S.E. of 4 determinations.

Compound	Conc. M	% Inhibition		
		4-Chloro- amphetamine	Low Na	Veratridine
H 102/09	$2.5 \times 10^{-6}$	$14 \pm 2$	—	—
	$2.5 \times 10^{-5}$	$48 \pm 2$	$61 \pm 6$	$59 \pm 8$
A 23189	$3.4 \times 10^{-6}$	$48 \pm 4$	$60 \pm 14$	$69 \pm 5$
Chlorimipramine	$2.9 \times 10^{-6}$	—	$56 \pm 11$	$43 \pm 3$
	$2.9 \times 10^{-5}$	$79 \pm 3$	$89 \pm 5$	$63 \pm 3$
Desipramine	$3.0 \times 10^{-6}$	$1 \pm 3$	$70 \pm 5$	$19 \pm 4$
	$3.0 \times 10^{-5}$	—	$9 \pm 3$	$40 \pm 5$
Millicaine	$2.9 \times 10^{-5}$	—	$8 \pm 4$	$82 \pm 3$

$0.05 > P > 0.01$

$0.01 > P > 0.001$

$P < 0.001$  (Student's *t* test).

with the exception that H 5-HT was omitted in the pre-incubation of the homogenate. The uptake of H 5-HT for a period of 5 min was examined at times corresponding to the start of the efflux experiment and after 60 min incubation at 37°C. The accumulation decreased from  $64.2 \pm 9.0$  ( $n = 5$ ) to  $16.8 \pm 1.8$  ( $n = 5$ ) pmol/g tissue/min incubation, i.e. the uptake after 60 min incubation was only 6% of that at zero time. Thus, the partially purified synaptosomes had lost a large part of their capacity to accumulate H 5-HT during the incubation.

#### *Effect of uptake inhibitors on the efflux of H 5-HT*

The effect of H 102/09, a selective inhibitor of the membranal 5-HT uptake (Ross *et al.* 1976), on the efflux of H 5-HT from the synaptosomes at 37°C was examined (Fig. 1 A). H 102/09 itself caused an increase of the spontaneous efflux, which was more marked after prolonged incubation. However, it significantly inhibited the initial increase of the efflux produced by 4-chloroamphetamine, by low external Na and by veratridine (Table I). Chlorimipramine (Shaskan and Snyder 1970) and A 23189 (unpublished observations) are two other potent inhibitors of the 5-HT uptake. As shown in Table I these compounds inhibited the efflux of H 5-HT induced by the various means examined. Desipramine which is a potent inhibitor of the NA uptake but a rather poor inhibitor of the 5-HT uptake (Ross and Renyi 1975) had much less effect in inhibiting the 5-HT efflux. At the higher concentration of desipramine examined the effect on the veratridine induced efflux was larger than on that evoked by low external Na. The same discrimination between the efflux evoked by veratridine and low external Na was obtained with the local anesthetic agent millicaine (Table I).



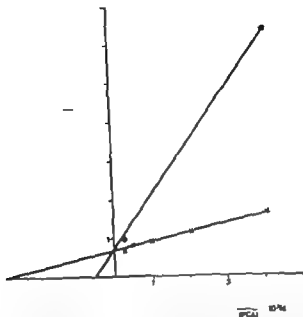


Fig. 2 Double reciprocal plot of the increase of the  $^3\text{H}$ -5-HT efflux caused by 4-chloroamphetamine (PCA). Synaptosomes are prepared and loaded with 11 5-HT as described in the legend of Fig. 1. The reaction time is 5 min. The velocity of efflux ( $v$ ) is determined as the difference of the efflux at  $37^\circ\text{C}$  and  $0^\circ\text{C}$  and was expressed in pmol/g/5min. The concentration of H 102/09 ( $\bullet$ ) was  $2.5 \cdot 10^{-6}$  M. Each value is the mean of 4 determinations.

#### Kinetics of the antagonism of H 102/09 on the 4-chloroamphetamine induced increase of 11-5-HT efflux

The kinetics of the antagonism of H 102/09 on the increased  $^3\text{H}$ -5-HT efflux produced by 4-chloroamphetamine was examined according to the method of Luxenberger and Burk (1974). The increased initial (5 min) efflux caused by 5 different concentrations of 4-chloroamphetamine ( $4 \cdot 10^{-6}$  M,  $3.9 \cdot 10^{-6}$  M) were determined in absence or presence of H 102/09 ( $5 \cdot 10^{-6}$  M). The result of an experiment is shown in Fig. 2. H 102/09 inhibited competitively the effect of 4-chloroamphetamine. The apparent  $K_m$  for 4-chloroamphetamine in increasing the efflux was  $4.6 \cdot 10^{-6}$  (mean of 2 determinations:  $5.4 \cdot 10^{-6}$  M and  $3.7 \cdot 10^{-6}$  M). The inhibitor constant ( $K_i$ ) for H 102/09 was  $1.1 \cdot 10^{-6}$  M (mean of  $1.5 \cdot 10^{-6}$  M and  $6.1 \cdot 10^{-6}$  M). Both these values are close to the corresponding  $K_i$  values for the inhibition of the 5-HT uptake in the same synaptosome preparation being  $1.2 \cdot 10^{-6}$  M for 4-chloroamphetamine and  $1.8 \cdot 10^{-6}$  M for H 102/09 (Fig. 3). The apparent  $K_m$  value for 5-HT as in these experiments is  $10^{-6}$  M.

#### Effect of ouabain on the increase in efflux produced by 4-chloroamphetamine

It is of interest to examine, if ouabain antagonized the increase in efflux produced by 4-chloroamphetamine, since if so, it should indicate that 4-chloroamphetamine is actively transported by a Na $^+$  dependent mechanism into the synaptosomes before it releases 5-HT.



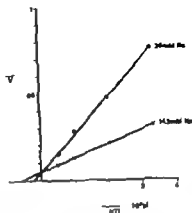


Fig. 4. Double reciprocal plot of the uptake of 5-HT in synaptosomes of rat cerebral cortex (non-reserpinized) at different  $\text{Na}^+$  concentrations. The incubation time was 3 min. The uptake velocity ( $V$ ) as determined as described in the legend of Fig. 3.

external  $\text{Na}^+$  concentration, whereas  $V_{\text{max}}$  was unchanged. The apparent  $K_m$  value for external  $\text{Na}^+$  concentration ( $1.7 \cdot 10^{-3} \text{ M}$ ) was in this experiment twice higher than that obtained with the more purified synaptosomes obtained from reserpinized rats.

### Discussion

The marked spontaneous efflux of H-5-HT from the cerebral cortical synaptosomes observed in this study is in accordance with the findings by Bogdanski *et al.* (1968). Since it was temperature dependent it is probably due to metabolic processes. Bogdanski *et al.* (1968) found that the labelled 5-HT decreased more rapidly than the endogenous 5-HT in the synaptosomes and proposed that dilution of the labelled amine with newly synthesized 5-HT as one reason for the decline in the labelled 5-HT. No attempt to test this hypothesis with an inhibitor of the 5-HT synthesis has been performed. Another possible mechanism is that the synaptosomes are unstable under the incubation conditions employed, are broken and release their contents out to the medium. The loss of a large part of the capacity of the synaptosomes to accumulate 5-HT after 60 min incubation at 37°C indicates that a part of the synaptosomes deteriorate in some way. This may at least partially contribute to the rapid spontaneous efflux of 5-HT observed at this temperature.

In spite of this spontaneous efflux of 5-HT very constant and reliable values were obtained when determining the initial 5 min efflux evoked by 4-chloroamphetamine, low external  $\text{Na}^+$  concentration and creatinine. Since this evoked 5-HT efflux was temperature dependent and is antagonized by uptake inhibitors it is probably an active outward transport of 5-HT. The observation that 11 102,09 which is a selective inhibitor of the neuronal 5-HT uptake (Rosa *et al.* 1976), accelerated the 5-HT efflux but antagonized the efflux evoked by various means appears somewhat conflicting. The acceleration of the efflux is understood, if it is supposed that a part of 5-HT released is taken up again into the synaptosomes by the membranous transport mechanism. The finding that the effect of 11 102,09 increased with time is in accordance with this interpretation, since the medium concentration of 5-HT increased and accordingly the uptake velocity. The antagonism of the evoked efflux by

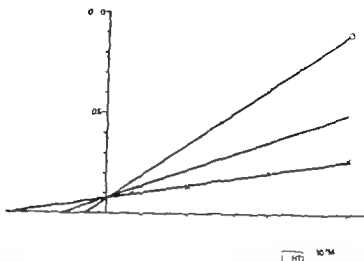


Fig. 3. Double reciprocal plot of the uptake of 5-HT by cerebral cortical synaptosomes from cerebellum. The incubation time was 5 min. The uptake velocity ( $V$ ) was determined as the difference at 37°C and 0°C and expressed in pmol/g/min. The concentration of 5-HT ( $10^{-6}$  M) was  $5 \times 10^{-6}$  M and the 4-chloroamphetamine ( $1 \times 10^{-6}$  M). Each value is the mean of 4 determinations.

intraneuronally. The synaptosomes were simultaneously incubated with ouabain in medium free of  $K^+$  and 4-chloroamphetamine at low concentration ( $1 \times 10^{-6}$  M) in order to be within an adequate concentration range for the potential uptake (Table II). The effect of this combination was larger than that of 4-chloroamphetamine and ouabain alone, which indicates an additive effect.

#### Kinetic determination of the Na<sup>+</sup> dependence of the 5-HT transport

Since both influx and efflux of 5-HT in synaptosomes are influenced by Na<sup>+</sup> (Bogdan *et al.* 1968) it was of interest to examine kinetically the nature of the Na<sup>+</sup> dependence. This was best performed by working with the uptake system, since the 5-HT concentration can then be properly controlled. In this experiment we used non-reserpinized rats and crude cortical homogenate obtained after low speed centrifugation (800  $g$ ). The determination of the uptake of 5-HT ( $3 \times 10^{-6}$  M– $2 \times 10^{-5}$  M) by the synaptosomes in the homogenate was performed in normal buffer and in buffer low in Na<sup>+</sup> (26 mM) by replacing Na<sup>+</sup> with 117 mM LiCl in order to keep the Cl<sup>-</sup> concentration unchanged. The double reciprocal plots (Fig. 4) show that the affinity of 5-HT for the uptake sites was decreased by the

TABLE II. The combined effect of ouabain ( $5 \times 10^{-6}$  M), no external  $K^+$  and 4-chloroamphetamine ( $1 \times 10^{-6}$  M) on the efflux of 5-HT from cerebral cortical synaptosomes of the rat. The incubation was performed for 5 min. Each value is mean  $\pm$  S.E. of 8 determinations.

	$\Delta$ Efflux pmol/g/min	S.E.	p
1. Ouabain 0 M	50.0	0.3	
2. 4-Chloroamphetamine	102.0	0.3	
3. 4-Chloroamphetamine + ouabain 0 M	128.0	0.1	0.001 ( $\chi^2$ )

an active transport of 4-chloroamphetamine into the synaptosomes and thereby preventing the intraneuronal release of H-5-HT. However, the finding that ouabain did not inhibit the effect of 4-chloroamphetamine but instead added its own effect to that of 4-chloroamphetamine indicates that 4-chloroamphetamine was not transported by the membranous 5-HT transfer system. Experiments with purified synaptosomes have also shown that H-4-chloroamphetamine has high affinity for the synaptosomes but no active, Na<sup>+</sup> dependent accumulation was observed (Rosa 1976 b).

The inhibition by 4-chloroamphetamine of the 5-HT uptake in the synaptosomes may not in fact be due to a real inhibition of the uptake but due to its 5-HT releasing effect. If the hypothesis of a reversible transport mechanism is correct the influx of 5-HT should equilibrate with the efflux at a much lower ratio between the inner and outer concentration of 5-HT than under normal condition, if the extravascular binding sites are occupied by 4-chloroamphetamine, which results in an elevated concentration of the free concentration of 5-HT. This hypothetical effect of 4-chloroamphetamine should accordingly be very similar to an inhibition of the uptake although the primary attack is not located to the transfer sites. This suggestion is in accordance with the observation that 4-chloroamphetamine was more or at least as potent in increasing the efflux than in inhibiting the uptake of 5-HT. According to the kinetic experiments H 102/09 inhibited competitively the increased efflux produced by 4-chloroamphetamine. Since the release by 4-chloroamphetamine of intraneuronally bound H-5-HT was probably directly related to the concentration of 4-chloroamphetamine the observed competitive nature of the effect of H 102/09 is not contradictory to the hypothesis that H 102/09 inhibited the outward transport of 5-HT. The almost identical K<sub>i</sub> values for H 102/09 in inhibiting the 5-HT uptake and the 4-chloroamphetamine induced 5-HT efflux further support the hypothesis of an identical mechanism for the uptake of 5-HT and the efflux of 5-HT evoked by 4-chloroamphetamine.

Alternative explanations of the data are possible. For instance, it can not be excluded that the influx and efflux are two separate mechanisms and that the inhibitors of the 5-HT uptake also antagonized the efflux mechanism due to similar structure requirements for both mechanisms.

## References

- BLANKSHAIN, T. P. and D. P. BOGDANOFF, Evidence for sodium dependent outward transport of the <sup>3</sup>H noradrenaline mediated by calyculin at the adrenergic synapse. Inhibition of transport by desipramine. *Life Sci.* 1977, 11, 867-876.
- BOGDANOFF, D. P., A. TAMARI and B. B. BROTH, Role of sodium, potassium, ouabain and reserpine in uptake, storage and metabolism of biogenic amines in synaptosomes. *Life Sci.* 1962, 7, 419-428.
- FELLER, R. W., C. W. HINES and J. MILLER, Lowering of brain serotonin level by chloroamphetamine. *British Pharmacol.* 1965, 11, 482-485.
- MCNICOLL, R. P., S. M. KUPFER, M. RIZZI and A. SYTHIAS, Actions and interactions of noradrenaline, tyramine and cocaine on norepinephrine of rabbit and left atria of guinea-pig and cat. *J. Pharmacol. exp. Ther.* 1963, 142, 39-58.
- HALL, R. W. and J. T. COYLE, The effects of anionic salts, temperature, and the alkaloids, reserpine and tetrachloro on the uptake of [<sup>3</sup>H]-dopamine into synaptosomes from rat striatum. *Molecular Pharmacol.* 1974, 10, 744-758.
- LEWIS, H. and D. BLISS, The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* 1934, 56, 633-644.

on the other hand understood if the transport of 5-HT under these experimental conditions occurs with the reversed uptake mechanism and this efflux accordingly inhibited by  $\Delta$  uptake inhibitors.

There are several observations supporting the hypothesis that the membranal 5-HT transport mechanism can be reversed. The  $\text{Na}^+$  dependence of the influx and efflux of 5-HT (Bogdanski *et al* 1968) indicates this possibility since several other  $\text{Na}^+$  dependent transport mechanisms are known to be reversible (Schultz and Curran 1970).  $\text{Na}^+$  influences the transports either by increasing the affinity of the solute for the uptake sites or by increasing the velocity of the transport. Observation in the present study indicates that the affinity of 5-HT is increased by  $\text{Na}^+$  which is in variance with the transport systems for noradrenaline (Sugrue and Shore 1971) and dopamine (Holz and Coyle 1974) in which the velocity of the transport but not the affinity appears to be increased. The explanation of the efflux of 5-HT by low external  $\text{Na}^+$  may accordingly be due to an equalization of the affinity on the two sides of the neurone membrane, which because of the much larger 5-HT concentration inside the synaptosomes results in the outward transport. Ouabain by inhibiting the  $\text{Na}^+$  pump increase the  $\text{Na}^+$  concentration in the synaptosomes and thereby the affinity of the 5-HT for the carrier sites on the inside of the membrane. Veratridine increases the  $\text{Na}^+$  permeability of excitable membranes (Ulbricht 1969) with the same result as with ouabain but with a more rapid onset of action. The finding that the local anesthetic agent miltiracaine discriminated between the 5-HT efflux induced by veratridine and low external  $\text{Na}^+$  indicates that the membrane stabilizing effect of the test compounds was not involved in the inhibition of the 5-HT efflux mechanism. Thus, the effect by miltiracaine on the veratridine evoked efflux could be explained by the antagonism of the depolarizing effect whereas the corresponding effect by the uptake inhibitors was probably due to the inhibition of the 5-HT transport. In accordance with this view a comparatively high concentration of desipramine, which is a rather poor inhibitor of the 5-HT uptake (Ross and Renyi 1975), had large effect on the veratridine evoked efflux, reflecting the membrane stabilizing action at the concentration. Indeed inhibition of the release of neurotransmitters induced by veratridine in synaptosomal preparations may become a valuable method for testing drugs on the membrane stabilizing action under identical or similar *in vitro* conditions as used in other assays. However the test compound must not inhibit the active outward transport of the transmitter examined.

The efflux induced by 4-chloroamphetamine can also be explained by the hypothesis of the reversed transport mechanism. This amino has high affinity for 5-HT neurones (Pletscher *et al* 1964, Fuller *et al* 1965). Since the vesicular storage mechanism of 5-HT was destroyed by reserpine, 4-chloroamphetamine, which because of its large lipophilicity readily passes membrane barriers, probably released 5-HT bound to some extravesicular binding sites in the nerve endings. The rise in the concentration of the soluble fraction of 5-HT in the synaptosomes results in increase in the outward transport of 5-HT since the rate of the transport is directly related to the concentration of 5-HT within the ranges of the transport capacity. The inhibition by the 5-HT uptake inhibitors of the efflux evoked by 4-chloroamphetamine, low external  $\text{Na}^+$  and high internal  $\text{Na}^+$  at the same concentration ranges support this hypothesis. It can be argued that the uptake inhibitors antagonized

active transport of 4-chloroamphetamine into the synaptosomes and thereby preventing intraneuronal release of H-5-HT. However the finding that ouabain did not inhibit the effect of 4-chloroamphetamine but instead added its own effect to that of 4-chloroamphetamine indicates that 4-chloroamphetamine was not transported by the membranous 5-HT transfer system. Experiments with purified synaptosomes have also shown that H-4-chloroamphetamine has high affinity for the synaptosomes but no active, Na<sup>+</sup> dependent accumulation was observed (Ross 1976 b).

The inhibition by 4-chloroamphetamine of the 5-HT uptake in the synaptosomes may not in fact be due to a real inhibition of the uptake but due to its 5-HT releasing effect. If the hypothesis of a reversible transport mechanism is correct the influx of 5-HT should equilibrate with the efflux at a much lower ratio between the inner and outer concentration of 5-HT than under normal condition, if the extra cellular binding sites are occupied by 4-chloroamphetamine, which results in an elevated concentration of the free concentration of 5-HT. This hypothetical effect of 4-chloroamphetamine should accordingly be very similar to an inhibition of the uptake although the primary attack is not located to the transfer sites. This suggestion is in accordance with the observation that 4-chloroamphetamine was more or at least as potent in increasing the efflux than in inhibiting the uptake of 5-HT. According to the kinetic experiments H 102/09 inhibited competitively the increased efflux produced by 4-chloroamphetamine. Since the release by 4-chloroamphetamine of intraneuronally bound <sup>3</sup>H-5-HT was probably directly related to the concentration of 4-chloroamphetamine the observed competitive nature of the effect of H 102/09 is not contradictory to the hypothesis that H 102/09 inhibited the outward transport of 5-HT. The almost identical  $K_{0.5}$  values for H 102/09 in inhibiting the 5-HT uptake and the 4-chloroamphetamine induced 5-HT efflux further support the hypothesis of an identical mechanism for the uptake of 5-HT and the efflux of 5-HT evoked by 4-chloroamphetamine.

Alternative explanations of the data are possible. For instance, it can not be excluded that the influx and efflux are two separate mechanisms and that the inhibitors of the 5-HT uptake also antagonized the efflux mechanism due to similar structure requirements for both mechanisms.

## References

- BLANKFORD, T. B. and D. P. BUDANIEL, Evidence for sodium dependent outward transport of the 11-norphenylephrine metabolite by calcium at the adrenergic synapse. Inhibition of transport by desipramine. *Life Sci.* 1977 11 867-876.
- BRADSHAW, D. E., A. THOMAS and B. B. BROTON, Role of sodium, potassium, ouabain and reserpine in uptake, storage and metabolism of biogenic amines in synaptosomes. *Life Sci.* 1968 11 419-428.
- FELLER, R. W., C. W. LINDS and J. MILLER, Lowering of brain serotonin level by chloroamphetamine. *British Pharmacol.* 1965 14 483-488.
- FRANKITT, R. F., S. M. KISHORE, M. RIKKI and A. SVETKEY, Actions and interactions of norphenylephrine, tyramine and cocaine on aortic strips of rabbit and left atria of guinea-pig and cat. *J. Pharmacol.* 1963 147 39-58.
- FRANKITT, R. W. and J. T. C. 12 The effects of anions, salts, temperature, and the alkaloids, tyramine and norephedrine on the uptake of [<sup>3</sup>H]-dopamine into synaptosomes from rat striatum. *Molecular Pharmacol.* 1972 12 746-758.
- LEVY, R. H. and D. R. 13 The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* 1924 46 670-684.

- PATON, D. M. Mechanism of efflux of noradrenaline from adrenergic nerves in rabbit *int. J. Pharmacol.* 1973 49 614-627.
- PLETSCHER, A., G. BARTHOLOM, H. BRUDERER, W. P. BURARD and K. F. GREY Chlorinated arylalkylamines affecting the cerebral metabolism of 5-hydroxytryptamine. *J. Pharmacol. exp. Ther.* 1964 145 344-358.
- ROSS, S. B., Structural requirements for uptake into catecholamine neurons. In: *The mechanisms of neuronal and extraneuronal transport of catecholamines*. Ed. D. M. Paton, 1976 a, pp. 67-93, Raven Press, New York.
- ROSS, S. B., Antagonism of the acute and long-term biochemical effects of 4-chloramphetamine on 5-HT neurons in the rat brain by inhibitors of the 5-hydroxytryptamine uptake. *Acta pharmacol. (Kb)* 1976 b, 39 456-476.
- ROSS, S. B. and A. L. RENYI, Inhibition of the uptake of tritiated 5-hydroxytryptamine in brain tissue. *Europ. J. Pharmacol.* 1969 7 270-277.
- ROSS, S. B. and A. L. RENYI, Uptake and metabolism of  $\beta$ -phenylethylamine and tyramine in mouse brain and heart slices. *J. Pharm. Pharmacol.* 1971 23 276-279.
- ROSS, S. B. and A. L. RENYI, Tricyclic antidepressant agents. I. Comparison of the uptake of  $^3$ H-norepinephrine and  $^{14}$ C-5-hydroxytryptamine in slices and crude synaptosome preparations of the midbrain-thalamus region of the rat brain. *Acta pharmacol. (Kbb.)* 1975, 36 382-394.
- ROSS, S. B., A. L. RENYI and B. BAUMGARTER, Cocaine-sensitive uptake of sympathomimetic amines in nerve tissue. *J. Pharm. Pharmacol.* 1968 20, 283-288.
- ROSS, S. B. and D. KELDER, Active transport of  $^3$ H-bretylum in the rat vas deferens *in vitro*. *Acta physiol. scand.* 1976, 97 209-221.
- ROSS, S. B., S.-O. ÖÖREN and A. L. RENYI, (Z)-Dimethylamino-1-(4-bromophenyl)-1-(3-pyridyl)propane (H 102/09) and new selective inhibitors of the neuronal 5-hydroxytryptamine uptake. *Acta pharmacol. (Kbb.)* 1976, 39 152-166.
- SCHULTZ, S. G. and P. P. CURRAN, Coupled transport of sodium and organic solutes. *Ph. med. Rev.* 1975, 50 637-718.
- SHANKS, E. and S. H. SNYDER, Kinetics of  $^3$ H-5-hydroxytryptamine uptake into synaptosomes in different regions of the brain. *J. Pharmacol. exp. Ther.* 1970 175 404-418.
- SNYDER, S. H. and J. T. COYLE, Regional differences in  $^3$ H-norepinephrine and  $^3$ H-dopamine uptake in rat brain homogenates. *J. Pharmacol. exp. Ther.* 1969 165 78-86.
- SUGRIZ, M. F. and P. A. STONE, Further evidence for sodium-dependent, optically specific and receptor-sensitive amine carrier mechanism at the adrenergic neuron. *J. Pharmacol. exp. Ther.* 1971 177 489-501.
- TINOA, N. B., G. F. WOOTEN, J. AXELROD and I. J. KOPIN, On the mechanism of release of norepinephrine from sympathetic nerves induced by depolarizing agents and sympathomimetic drugs. *Mol. Pharmacol.* 1975 11 10-18.
- TAENDELINBURG, U. Classification of sympathomimetic amines. In: *Catecholamines*. Ed. H. Blasch and E. Muschöhl, 1972, pp. 336-360. Springer Verlag Berlin, Heidelberg, New York.
- ULBRICHT, W. The effect of carindane on excitable membranes of nerve and muscle. *Drugs Phys.* 1969 61 18-71.



## Preferential Uptake of $^3\text{H}$ - $\alpha$ Aminoisobutyric Acid Into Mouse Uterine Tissue during Early Pregnancy

By

INGOER LINDQVIST, OVE NILSSON\* and GUDNAR RÖNQVIST†

Received 11 May 1976

### Abstract

LINDQVIST I., O. NILSSON and G. RÖNQVIST. Preferential uptake of  $^3\text{H}$ - $\alpha$ -aminoisobutyric acid into mouse uterine tissue during early pregnancy. *Acta physiol. scand.* 1977 99: 37-41.

The uptake of  $^3\text{H}$ -AIB into uterine tissue and some other organs was studied during early implantation. Mice in experimentally delayed implantation were given estrogen and  $^3\text{H}$ -AIB at different time intervals. Mice in delayed implantation given only  $^3\text{H}$ -AIB displayed low transport rate into the uterine tissue comparable with that for the diaphragm muscle. However, if the estrogen injection preceded that of the  $^3\text{H}$ -AIB by 4 to 8 h the transport capacity increased by a factor of at least 4 times, while the uptake for the diaphragm muscle remained low. Under such conditions  $^3\text{H}$ -AIB-accumulation into uterine tissue was increased for at least 4 h of *in vivo* incubation with the labeled amino acid.

The rate of amino acid transport appears to be closely correlated to the rate of protein synthesis of both normal and malignant tissues (Riggs 1964, Griffiths 1967). Glucocorticoids, which are catabolic hormones, work antagonistically to the transport of amino acids into skeletal muscle (Riggs 1964, Christensen 1975), while insulin and growth hormone, for example, which are both anabolic hormones and promote protein synthesis accelerate amino acid transport into several tissues (Riggs 1964). Also estrogens promote growth in particular tissues (Noall *et al.* 1957, Noall and Allen 1961) and consequently McCorquodale and Mueller (1958) observed an increased activity of amino acid-activating enzyme in the uterus already 3 h after treatment with estrogen. An early  $\gamma$ -induced protein, after exposure of uterus to estradiol, has recently been detected among the soluble proteins (Pennequin, Robel and Bauleux 1975).

The capacity to transport amino acids is probably also higher since the level of free amino acid is higher in the more rapidly growing tissues (Noall *et al.* 1958). This is tested by using  $\alpha$ -aminoisobutyric acid, AIB, as an amino acid substrate since this amino acid is not metabolized. As expected, Noall *et al.* (1958) found a 3-fold increase in the uterine accumulation of AIB upon administration of estradiol, and Riggs and Pan (1972) observed an incre-

*in vitro* transport of AIB into the estrogen-primed uterus. Other studies also indicate influence *in vitro* by estrogen on AIB uptake and transport (Riggs *et al.* 1968, Walters *et al.* 1975).

Since early pregnancy specifically the time of blastocyst implantation, is associated with an increase in the concentration of estrogen we questioned whether the capacity of AIB transport is increased as well. If so this might have implications for the nutrition of the blastocyst at trophoblast attachment and invasion. Therefore, the aim of the present investigation was, firstly to examine the characteristics of AIB transport *in vivo* into some organs of mice, conditioned for experimentally delayed implantation (Humphrey 1967), and secondly to examine whether the capacity of AIB transport *in vivo* into the uterus and some other organs increased after the initiation of implantation by estrogen.

### Materials and methods

$^3\text{H}$ - $\alpha$ -aminoisobutyric acid ( $^3\text{H}$ -AIB), Protosol and Aquasol were purchased from NEN Chemicals GmbH Dreieichenhain, W. Germany and  $\alpha$ -aminoisobutyric acid from Calbiochem, San Diego, CA. Progesterone was purchased from Iliapharm, Ramat-Gan, Israel and estradiol from AB Leo, Helsingborg, Sweden. All chemicals used were analytical grade. Radioactivity was measured in a Nuclear Chet Undux II Liquid Scintillation Counter.

Adult white female mice (N.M.R.I., National Medical Research Institute, Bethesda, USA) were mated and the day the vaginal plug was seen was recorded as day 1. They were spayed day 3 and given the first dose of 1 mg of progesterone in 0.04 ml peanut oil subcutaneously the same day. The animals were kept in delayed implantation by daily injections of progesterone. Implantation was initiated by a subcutaneous injection of 0.1 g estradiol-17 $\beta$  in 0.1 ml propylene glycol. Generally 4.5  $\mu\text{Ci}$   $^3\text{H}$ -AIB in 0.1 ml of a 40 mM AIB solution, made isotonic with NaCl, was given i.p. in the tail to each animal. Blood samples were drawn from the cut tail and directly transferred to the liquid scintillation bottles. Tissue samples were washed on an ice-cold Krebs-Ringer bicarbonate buffer freshly bubbled with a gas mixture of 93.5%  $\text{O}_2$  and 6.5%  $\text{CO}_2$  to give a pH of 7.4 and lightly blotted on tissue paper before being transferred to the bottles. The animals were sacrificed by giving them Nembutal before the tissue samples were taken.

Samples were transferred into weighed liquid scintillation counting bottles, weighed and dissolved in 1 ml Protosol at 50°C. The solutions were mixed with 50% conc. acetic acid (to minimize fluorescence) and 10 ml of Aquasol. Samples were then counted and counts corrected for self absorption.

### Results

The clearance of  $^3\text{H}$ -AIB was established by determining the time at which the amino acid concentration curve of the blood crossed that of the urine. The labeled amino acid was i.v. administered to the animals which were then sacrificed at different times between 5 and 20 min after the injection of the labeled amino acid. Fig. 1 shows that a peak value in blood was reached after 15 min and that the declining blood curve crossed the rising curve reflecting the urine concentration of  $^3\text{H}$ -AIB after 20 min. The curve profiles of both blood and urine were quite similar in 4 different experiments with animals in delayed implantation as well as with estrogen-treated animals. It was therefore convenient to sacrifice the animals 20 min after the i.v. administration of  $^3\text{H}$ -AIB during the next series of experiments.

The distribution of  $^3\text{H}$ -AIB into the uterus and some other tissues during early implantation was examined in 4 groups, each containing 3 mice in experimentally delayed implantation. Animals of one group (controls) were given only  $^3\text{H}$ -AIB 20 min before death. The

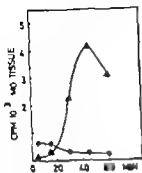


Fig. 1

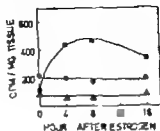


Fig. 2

Fig. 1.  $^{14}\text{C}$  AIB concentration in blood and recovery in uterus at different times after intravenous administration.  $\circ$   $\circ$  blood,  $\triangle$   $\triangle$  uterus.

Fig. 2. Recovery of  $^{14}\text{C}$  AIB in blood ( $\circ$ — $\circ$ ), diaphragm muscle ( $\triangle$ — $\triangle$ ) and uterine tissue ( $\square$ — $\square$ ) at different stages of implantation induced by estrogen. Stand. dev.  $\max \pm 40$  cpm.

most of the other 3 groups were given an injection of estrogen, and  $^{14}\text{C}$ -AIB was administered 20 min before death, 4, 8 and 16 h respectively after the estrogen injection. Blood, diaphragm muscle and uterus were examined.

The level of  $^{14}\text{C}$  AIB in the blood was about the same between 0 and 16 h after the injection of estrogen. The  $\text{in vivo}$  uptake by the diaphragm muscle was quite low and independent of the time after the estrogen injection. The  $\text{in vivo}$  uptake by the uterine tissue, on the contrary displayed a characteristic profile with a low uptake of  $^{14}\text{C}$ -AIB for animals in delayed implantation (controls), marked increase at 4 and 8 h after the initiation of implantation by estrogen and a slight decrease at 16 h as compared with the values for 4 and 8 h (Fig. 2).

The retention of  $^{14}\text{C}$  AIB was measured in blood, diaphragm muscle, brain and uterus. Mice were given the labeled amino acid 4 h after the injection of estrogen and the tissue concentrations were measured after different *in vitro* incubation times with the labeled amino acid substrate. 5 groups, each containing at least 3 animals in experimentally delayed implantation were used. The animals were sacrificed 1/3, 4, 16, 24 and 48 h after the injection of  $^{14}\text{C}$  AIB (Fig. 1).

Although the blood concentration of the labeled amino acid was declining already at 20 min after the administration (cf. Fig. 1) and remained low throughout the experiment, the  $^{14}\text{C}$  AIB was retained in the uterus for several hours. Still at 16 h the uterine concentration of the labeled amino acid was higher than the 20 min blood value. Also after 16 h the blood value was 7 times lower than that of the uterus. Diaphragm muscle showed a slow accumulation of  $^{14}\text{C}$  AIB during at least the first 4 h and retained the labeled amino acid for several hours. The brain displayed a somewhat longer accumulation time and a higher uptake than the diaphragm muscle. The uptake of  $^{14}\text{C}$  AIB was nevertheless much lower in these tissues than in the uterus. The prolonged uptake process into brain tissue *in vivo* might be explained by the existence of specific carrier systems for amino acids in the blood-brain barrier (Oldendorf 1973) with a relatively slow transport rate for AIB (Oldendorf 1973).

*in vitro* transport of AIB into the estrogen primed uterus. Other studies also indicate influence *in vitro* by estrogen on AIB uptake and transport (Riggs *et al.* 1968, Walters *et al.* 1975).

Since early pregnancy specifically the time of blastocyst implantation, is associated with an increase in the concentration of estrogen we questioned whether the capacity of AIB transport is increased as well. If so, this might have implications for the nutrition of the blastocyst at trophoblast attachment and invasion. Therefore, the aim of the present investigation was, firstly to examine the characteristics of AIB transport *in vivo* into some organs of mice, conditioned for experimentally delayed implantation (Humphrey 1967), and secondly to examine whether the capacity of AIB transport *in vivo* into the uterus and so other organs increased after the initiation of implantation by estrogen.

### Materials and methods

<sup>14</sup>C- $\alpha$ -aminoisobutyric acid (<sup>14</sup>C AIB), Protosol and Aquasol were purchased from NEN Chem. GmbH Dreieichenb., W. Germany and  $\alpha$ -aminoisobutyric acid from Calbiochem, San Diego, U.S.A. Progesterone was purchased from Ilabpharm, Ramat-Gan, Israel and estradiol from AB Leo, Ulfsholm, Sweden. All chemicals used were analytical grade. Radioactivity was measured in Nuclear Chicago II Liquid Scintillation Counter.

Adult white female mice (N.M.R.I., Naval Medical Res. Inst. Bethesda, USA) were mated and the vaginal plug was seen was recorded as day 1. They were spayed on day 3 and given the first dose of 1 mg of progesterone in 0.04 ml peanut oil subcutaneously the same day. The animals were kept during implantation by daily injections of progesterone. Implantation was initiated by a subcutaneous injection of 0.1 g estradiol-17 $\beta$  in 0.1 ml propyleneglycol. Generally 4.25  $\mu$ Ci <sup>14</sup>C AIB (0.1 ml of a 40 mM solution, made isotonic with NaCl, was given i.p. in the tail to each animal. Blood samples were drawn from the cut tail and directly transferred to the liquid scintillation bottles. Tissue samples were aspired into ice-cold Krebs-Ringer bicarbonate buffer freshly bubbled with a gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub> to give a pH of 7.4 and lightly blotted on tissue paper before being transferred to the bottles. The animals were sacrificed by giving them Nembutal before the tissue samples were taken.

Samples were transferred into weighed liquid scintillation counting bottles, weighed and dissolved in 1 ml Protosol at 50°C. The solutions were mixed with 50  $\mu$ l conc. acetic acid (1:1 measure for measure "sensitivity") and 10 ml of Aquasol. Samples were then counted and counts corrected for self-absorption.

### Results

The clearance of <sup>14</sup>C AIB was established by determining the time at which the amino acid concentration curve of the blood crossed that of the urine. The labeled amino acid was administered to the animals which were then sacrificed at different times between 5 and 20 min after the injection of the labeled amino acid. Fig. 1 shows that a peak value in blood was reached after 15 min and that the declining blood curve crossed the rising curve reflecting the urine concentration of <sup>14</sup>C AIB after 20 min. The curve profiles of both blood and urine were quite similar in 4 different experiments, with animals in delayed implantation as well as with estrogen treated animals. It was therefore convenient to sacrifice the animals 20 min after the *iv* administration of <sup>14</sup>C AIB during the next series of experiments.

The distribution of <sup>14</sup>C AIB into the uterus and some other tissues during early implantation was examined in 4 groups, each containing 3 mice in experimentally delayed implantation. Animals of one group (controls) were given only <sup>14</sup>C AIB 20 min before death. The

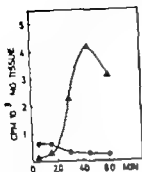


Fig. 1

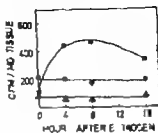


Fig. 2

Fig. 1  $^{14}\text{C}$ -AIB concentration in blood and recovery in urine at different times after intravenous administration.  $\circ$ — $\circ$  blood,  $\triangle$ — $\triangle$  urine.

Fig. 2 Recovery of  $^{14}\text{C}$ -AIB in blood ( $\circ$ — $\circ$ ), diaphragm muscle ( $\triangle$ — $\triangle$ ) and uterine tissue ( $\square$ — $\square$ ) at different stages of implantation initiated by estrogen. Stool excretion  $\pm 40$  cpm.

moes of the other 3 groups were given an injection of estrogen, and  $^{14}\text{C}$ -AIB was administered 30 min before death, 4, 8 and 16 h respectively after the estrogen injection. Blood, diaphragm muscle and uterus were examined.

The level of  $^{14}\text{C}$ -AIB in the blood was about the same between 0 and 16 h after the injection of estrogen. The *in vivo* uptake by the diaphragm muscle was quite low and independent of the time after the estrogen injection. The *in vivo* uptake by the uterine tissue, on the contrary, displayed a characteristic profile with a low uptake of  $^{14}\text{C}$ -AIB for animals in delayed implantation (controls), marked increase at 4 and 8 h after the initiation of implantation by surgery and a slight decrease at 16 h as compared with the values for 4 and 8 h (Fig. 2).

The retention of  $^{14}\text{C}$ -AIB was measured in blood, diaphragm muscle, brain and uterus. Mice were given the labeled amino acid 4 h after the injection of estrogen and the tissue concentrations were measured after different *in vivo* incubation times with the labeled amino acid substrate. 5 groups, each containing at least 3 animals in experimentally delayed implantation were used. The animals were sacrificed 1/3, 4, 16, 24 and 48 h after the injection of  $^{14}\text{C}$ -AIB (Fig. 1).

Although the blood concentration of the labeled amino acid was declining already at 20 min after the administration (cf. Fig. 1) and remained low throughout the experiment, the  $^{14}\text{C}$ -AIB was retained in the uterus for several hours. Still at 16 h the uterine concentration of the labeled amino acid was higher than the 20 min blood value. Also after 16 h the blood value was 7 times lower than that of the uterus. Diaphragm muscle showed a slow accumulation of  $^{14}\text{C}$ -AIB during at least the first 4 h and retained the labeled amino acid for several hours. The brain displayed somewhat longer accumulation time and a higher uptake than the diaphragm muscle. The uptake of  $^{14}\text{C}$ -AIB was nevertheless much lower in these tissues than in the uterus. The prolonged uptake process into brain tissue *in vivo* might be explained by the existence of specific carrier systems for amino acids in the blood-brain barrier (Oldendorf 1973) with relatively slow transport rate for AIB (Oldendorf 1971).

*In vitro* transport of AIB into the estrogen primed uterus. Other studies also indicate influence *in vitro* by estrogen on AIB uptake and transport (Riggs *et al* 1968, Walters, 1975)

Since early pregnancy specifically the time of blastocyst implantation, is associated an increase in the concentration of estrogen we questioned whether the capacity of transport is increased as well. If so this might have implications for the nutrition of blastocyst at trophoblast attachment and invasion. Therefore, the aim of the present investigation was, firstly to examine the characteristics of AIB transport *in vivo* into some of mice, conditioned for experimentally delayed implantation (Humphrey 1967) secondly to examine whether the capacity of AIB transport *in vivo* into the uterus and other organs increased after the initiation of implantation by estrogen.

### Materials and methods

$^4\text{C}$   $\alpha$ -aminoisobutyric acid (1  $^4\text{C}$  AIB), Protocol and Aquasol were purchased from NEN Chem GmbH Dreieichenbain, W. Germany and  $\alpha$ -aminoisobutyric acid from Calbiochem, San Diego, I. Progesterone was purchased from Ikapharm, Ramat-Gan, Israel and estradiol from AB Leo Hålsjö, Sweden. All chemicals used were analytical grade. Radioactivity was measured in a Nuclear Ch. Unix II Liquid Scintillation Counter.

Adult white female mice (N M R.I. Nav 1 Medical Res. Inst. Bethesda, USA) were mated and the vaginal plug was seen was recorded as day 1. They were spayed on day 3 and given the first dose of progesterone 0.04 ml peanut oil subcutaneously the same day. The animals were kept in delayed implantation by daily injections of progesterone. Implantation was initiated by a subcutaneous injection of 0.1 g estradiol 17 $\beta$  in 0.1 ml propyleneglycol. Generally 4.25  $^4\text{C}$  AIB in 0.1 ml of a 40 mM solution made isotonic with NaCl, was given i. i. the tail to each animal. Blood samples were taken from the cut tail and directly transferred to the liquid scintillation bottles. Tissue samples were excised from the cold Krebs-Ringer bicarbonate buffer freshly bubbled with a gas mixture of 91.5  $\text{O}_2$  and 8.5  $\text{CO}_2$  gave a pH of 7.4, and lightly blotted on tissue paper before being transferred to the bottles. Animals were sacrificed by giving them Nembutal before the tissue samples were taken.

Samples were transferred into weighed liquid scintillation counting bottles, weighed and dissolved in 1 ml Protocol at 50  $^{\circ}\text{C}$ . The solutions were mixed with 50  $\mu\text{l}$  concentrated acetic acid (to increase "sensitivity") and 10 ml of Aquasol. Samples were then counted and counts corrected for self absorption.

### Results

The clearance of  $^4\text{C}$  AIB was established by determining the time at which the arterial concentration curve of the blood crossed that of the urine. The labeled amino acid was administered to the animals which were then sacrificed at different times between 5 and 20 min after the injection of the labeled amino acid. Fig. 1 shows that a peak value in blood was reached after 15 min and that the declining blood curve crossed the rising curve reflecting the urine concentration of  $^4\text{C}$  AIB after 20 min. The curve profiles of both blood and urine were quite similar in 4 different experiments with animals in delayed implantation as well as with estrogen-treated animals. It was therefore convenient to sacrifice the animals 20 min after the *in vivo* administration of  $^4\text{C}$  AIB during the next series of experiments.

The distribution of  $^4\text{C}$  AIB into the uterus and some other tissues during early implantation was examined in 4 groups, each containing 3 mice in experimentally delayed implantation. Animals of one group (controls) were given only  $^4\text{C}$  AIB 20 min before death.

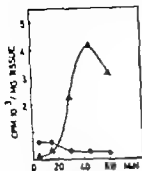


Fig. 1

Fig. 1.  $^{14}\text{C}$  AIB concentration in blood and recovery in uterus at different times after estrone treatment.  $\bigcirc$ — $\bigcirc$  blood,  $\triangle$ — $\triangle$  uterus.

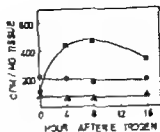


Fig. 2

Fig. 2. Recovery of  $^{14}\text{C}$  AIB in blood ( $\bigcirc$ — $\bigcirc$ ), diaphragm muscle ( $\triangle$ — $\triangle$ ) and uterine tissue ( $\square$ — $\square$ ) at different stages of implantation induced by estrogen. Stage day max. 48 cpm.

rest of the other 3 groups were given an injection of estrogen, and  $^{14}\text{C}$  AIB was administered 30 min before death, 4, 8 and 16 h respectively after the estrogen injection. Blood, diaphragm muscle and uterus were examined.

The level of  $^{14}\text{C}$  AIB in the blood was about the same between 0 and 16 h after the injection of estrogen. The uptake by the diaphragm muscle was quite low and independent of the time after the estrogen injection. The uterine uptake by the uterine tissue, on the contrary displayed a characteristic profile with a low uptake of  $^{14}\text{C}$  AIB for animals in delayed implantation (controls), marked increase at 4 and 8 h after the initiation of implantation by estrogen and a slight decrease at 16 h as compared with the values for 4 and 8 h (Fig. 2).

The retention of  $^{14}\text{C}$  AIB was measured in blood, diaphragm muscle, brain and uterus. Mice were given the labeled amino acid 4 h after the injection of estrogen and the tissue concentrations were measured after different incubation times with the labeled amino acid substrate. 5 groups, each containing at least 3 animals (in experimentally delayed implantation) were used. The animals were sacrificed 1/3, 4, 16, 44 and 48 h after the injection of  $^{14}\text{C}$  AIB (Fig. 1).

Although the blood concentration of the labeled amino acid was declining already at 20 min after the administration (cf. Fig. 1) and remained low throughout the experiment, the  $^{14}\text{C}$  AIB was retained in the uterus for several hours. Still at 16 h the uterine concentration of the labeled amino acid was higher than the 20 min blood value. Also after 16 h the blood value was 7 times lower than that of the uterus. Diaphragm muscle showed a slow accumulation of  $^{14}\text{C}$  AIB during at least the first 4 h and retained the labeled amino acid for several hours. The brain displayed a somewhat longer accumulation time and a higher uptake than the diaphragm muscle. The uptake of  $^{14}\text{C}$  AIB was nevertheless much lower in these tissues than in the uterus. The prolonged uptake process in brain tissue may be explained by the existence of specific carrier systems for amino acids in the blood-brain barrier (Oldendorf 1973) with a relatively slow transport rate for AIB (Oldendorf

*in vitro* transport of AIB into the estrogen-primed uterus. Other studies also indicate influence *in vitro* by estrogen on AIB uptake and transport (Riggs *et al.* 1968, Walters *et al.* 1975).

Since early pregnancy specifically the time of blastocyst implantation is associated with an increase in the concentration of estrogen we questioned whether the capacity of AIB transport is increased as well. If so this might have implications for the nutrition of the blastocyst at trophoblast attachment and invasion. Therefore, the aim of the present investigation was, firstly to examine the characteristics of AIB transport *in vivo* into some organs of mice, conditioned for experimentally delayed implantation (Humphrey 1967), and secondly to examine whether the capacity of AIB transport *in vitro* into the uterus and some other organs increased after the initiation of implantation by estrogen.

### Materials and methods

$^4\text{C}$ - $\alpha$ -aminoisobutyric acid ( $1\text{-}^{14}\text{C}$  AIB), Protocol and Aquasol were purchased from NEN Chemical GmbH, Dreieichenhain, W. Germany and  $\alpha$ -aminoisobutyric acid from Calbiochem, San Diego, U.S.A. Progesterone was purchased from Ikapharm, Ramat-Gan, Israel and estradiol from AB Leo, Helsingborg, Sweden. All chemicals used were analytical grade. Radioactivity was measured in a Nuclear Chicago 11 Liquid Scintillation Counter.

Adult white female mice (N.M.R.I., National Medical Research Institute, Bethesda, USA) were mated and the day the vaginal plug was seen was recorded as day 1. They were spayed on day 3 and given the first dose of 1 mg of progesterone in 0.04 ml peanut oil subcutaneously the same day. The animals were kept in delayed implantation by daily injections of progesterone. Implantation was initiated by a subcutaneous injection of 0.1 g estradiol 17 $\beta$  in 0.1 ml propylene glycol. Generally 4.25  $\mu\text{Ci}$   $^4\text{C}$  AIB in 0.1 ml of a 40 mM AIB solution, made isotonic with NaCl, was given i.v. in the tail to each animal. Blood samples were drawn from the cut tail and directly transferred to the liquid scintillation bottles. These samples wereashed and in ice-cold Krebs Ringer bicarbonate buffer freshly bubbled with a gas mixture of 93.5%  $\text{O}_2$  and 6.5%  $\text{CO}_2$  to give a pH of 7.4, and lightly blotted on tissue paper before being transferred to the bottles. The animals were sacrificed by giving them Nembutal before the tissue samples were taken.

Samples were transferred into weighed liquid scintillation counting bottles, weighed and desolved overnight in 1 ml Protocol at  $50^\circ\text{C}$ . The solutions were mixed with 50  $\mu\text{l}$  conc. acetic acid (to minimize "quench sensitivity") and 10 ml of Aquasol. Samples were then counted and countings corrected for self absorption.

### Results

The clearance of  $^4\text{C}$  AIB was established by determining the time at which the arterial acid concentration curve of the blood crossed that of the urine. The labeled amino acid was i.v. administered to the animals which were then sacrificed at different times between 5 and 20 min after the injection of the labeled amino acid. Fig. 1 shows that a peak value in blood was reached after 15 min and that the declining blood curve crossed the rising curve reflecting the urine concentration of  $^4\text{C}$  AIB after 20 min. The curve profiles of both blood and urine were quite similar in 4 different expts. with animals in delayed implantation as well as with estrogen-treated animals. It was therefore convenient to sacrifice the animals 20 min after the i.v. administration of  $^4\text{C}$  AIB during the next series of expts.

The distribution of  $^4\text{C}$  AIB into the uterus and some other tissues during early implantation was examined in 4 groups, each containing 3 mice in experimentally delayed implantation. Animals of one group (controls) were given only  $^4\text{C}$  AIB 20 min before death. The



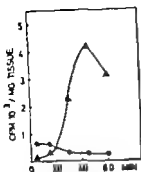


Fig. 1

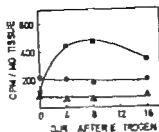


Fig. 2

Fig. 1  $^{14}\text{C}$ -AIB concentration in blood and recovery in urine at different times after intravenous administration.  $\bullet$ — $\bullet$  blood  $\blacktriangle$ — $\blacktriangle$  urine.

Fig. 2 Recovery of  $^{14}\text{C}$ -AIB in blood ( $\bullet$ — $\bullet$ ), diaphragm muscle ( $\blacktriangle$ — $\blacktriangle$ ) and uterine tissue ( $\blacksquare$ — $\blacksquare$ ) at different stages of implantation induced by estrogen. Stand. dev. max  $\pm$  40 cpm.

Each of the other 3 groups were given an injection of estrogen, and  $^{14}\text{C}$  AIB was administered 20 min before death, 4, 8 and 16 h respectively after the estrogen injection. Blood, diaphragm muscle and uterus were examined.

The level of  $^{14}\text{C}$  AIB in the blood was about the same between 0 and 16 h after the injection of estrogen. The *in vivo* uptake by the diaphragm muscle was quite low and independent of the time after the estrogen injection. The *in vivo* uptake by the uterine tissue, on the contrary displayed a characteristic profile with a low uptake of  $^{14}\text{C}$  AIB for animals in delayed implantation (controls), marked increase at 4 and 8 h after the initiation of implantation by estrogen and a slight decrease at 16 h as compared with the values for 4 and 8 h (Fig. 2).

The retention of  $^{14}\text{C}$  AIB was measured in blood, diaphragm muscle, brain and uterus. Mice were given the labeled amino acid 4 h after the injection of estrogen and the tissue concentrations were measured after different *in vivo* incubation times with the labeled amino acid substrate. 5 groups, each containing at least 3 animals in experimentally delayed implantation were used. The animals were sacrificed 1/3, 4, 16, 4 and 48 h after the injection of  $^{14}\text{C}$ -AIB (Fig. 3).

Although the blood concentration of the labeled amino acid was declining already at 30 min after the administration (cf. Fig. 1) and remained low throughout the experiment, the  $^{14}\text{C}$  AIB was retained in the uterus for several hours. Still at 16 h the uterine concentration of the labeled amino acid was higher than the 30 min blood value. Also after 16 h the blood value was 7 times lower than that of the uterus. Diaphragm muscle showed a slow accumulation of  $^{14}\text{C}$  AIB during at least the first 4 h and retained the labeled amino acid for several hours. The brain displayed a somewhat longer accumulation time and higher value than the diaphragm muscle. The uptake of  $^{14}\text{C}$  AIB was nevertheless much lower in these tissues than in the uterus. The prolonged uptake process into brain tissue may be explained by the existence of specific carrier systems for amino acids in the blood-brain barrier (Oldendorf 1973) with a relatively slow transport rate for AIB (Oldendorf

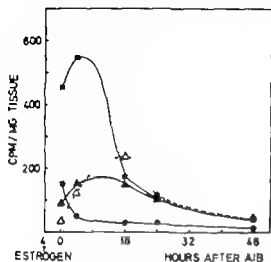


Fig. 3. Retention time of  $^{14}\text{C}$  AIB in blood (●—●), diaphragm muscle (○—○), brain (△---△) and uterine tissue (■—■).  $^{14}\text{C}$  AIB was administered 4 h after estrogen initiation of implantation. *Stand. dev.* =  $\text{max} \pm 50$  cpm.

### Discussion

Mice in delayed implantation which in addition to progesterone had received estrogen 4 or 8 h before being given  $^{14}\text{C}$  AIB i.v. displayed a high uterine uptake compared with mice not having received estrogen. Furthermore this uptake was preferential for the uterine tissue compared with some other tissues. Since the  $^{14}\text{C}$  AIB transport was considerably lower when only  $^{14}\text{C}$  AIB was given pretreatment *in vivo* with estrogen is necessary for the enhanced  $^{14}\text{C}$  AIB-accumulation. This is in agreement with the findings of Riggs *et al.* (1968) for the *in vitro* uptake of AIB in immature rat uterus after *in vitro* preincubation with estradiol. Since the estrogen effect is not momentaneous it seems probable that the hormone might induce a *de novo* synthesis of AIB-carriers in the plasma membranes of uterine tissue. Such a proposal can be further supported by the determination of the  $V_{\text{max}}$  parameter and Riggs *et al.* (1968) have shown that in their experimental system  $V_{\text{max}}$  increased while  $K_m$  remained unchanged by the treatment with estradiol.

AIB has been shown to be transported mainly by the route defined as the system A by Oxender and Christensen (1963). Several other amino acids as alanine, methionine and nor-leucine are mainly transported by this route in different cells. Characteristical features for this system are the Na<sup>+</sup>-dependency and transport against a concentration gradient. This means necessarily that the exit rate by this system is slower than the entrance rate (Christensen and Handlogten 1968) and that the carrier operates asymmetrically. By this system it is therefore possible to augment the free intracellular amino acid pool while an exchange system can only influence the qualitative composition of the intracellular amino acid pool without enhancing the net uptake. Such an asymmetry has been shown to exist in slices of newborn rat kidney cortex while absent in adult cortex (Webber 1968, Webber and Cairns 1968).

The strict Na<sup>+</sup>-dependence of the AIB-uptake into uterine tissue can naturally not be demonstrated by *in vitro* expts. However Riggs *et al.* (1972) have shown that the uptake of AIB into the estrogen primed rat uterus *in vitro* increased regularly with increasing Na<sup>+</sup> amounts in the incubation buffer. Thus we have reasons to believe that the system A is operating also *in vivo* in mice.

During activation for implantation the first 8 h are a period when system A amino acids are supplied from the blood into the uterine tissue at a high degree. Perhaps this accumulation of amino acids in the uterine tissue can be exploited also by the developing blastocyst. If so, AIB should accumulate also in the blastocyst. This possibility is presently being tested.

The investigation is supported by grants from the Swedish Medical Research Council, project no B76-1X-223-1-A and no 12X 70. Technical assistant is Mrs Barbro Elmarsson.

## References

1. AUSTIN, H. N. *Biological Transport*, 2nd ed. 1975. W. A. Benjamin, Inc., Reading, Mass., U.S.A.
2. AUSTIN, H. N. and M. E. HANLOTTON, Modes of Mediated Exocytosis of Amino Acids from the Ehrlich Ascites Tumor Cells. *J. Biol. Chem.* 1968, 243, 5428-5438.
3. GERTZ, J. B. and S. L. PIS. The uptake of amino acids by mouse cells (strain L8) during growth in batch culture and chemostat culture: the influence of cell growth rate. *Proc. roy. Soc. B* 1967, 162, 421-438.
4. HANLOTTON, M. E. W. The induction of implantation in the mouse after ovariectomy. *Surgery* 1967, 70, 581-601.
5. MCCOY, D. L. and G. E. MULLER, Effect of estradiol on the level of amino acid-activating enzymes in the rat uterus. *J. Biol. Chem.* 1958, 233, 31-42.
6. NOLLE, M. W. and W. M. ALLEN, Early stimulation by estradiol of amino acid penetration in rabbit uterus. *J. Biol. Chem.* 1961, 236, 2987-2990.
7. NOLLE, M. W., T. R. RIGGS, L. M. WALLER and H. N. CHRISTENSEN, Endocrine control of amino acid transfer. *Science* 1957, 126, 1002-1005.
8. OLSSON, W. H. Brain uptake of radiolabelled amino acids, amines and hexoses after arterial injection. *Amer. J. Physiol.* 1971, 221, 1629-1639.
9. OLSSON, W. H. Stereospecificity of blood-brain barrier permeability to amino acids. *Amer. J. Physiol.* 1973, 224, 967-969.
10. OLSSON, D. L. and H. N. CHRISTENSEN, Distinct producing systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* 1963, 238, 3486-3499.
11. ROBERTS, P. P., ROBERT, P. and E. E. BALGUTH, Steroid-induced early protein synthesis in rat uterus and prostate. *Europ. J. Biochem.* 1975, 60, 157-165.
12. RICE, T. R. *Action of hormones on molecular processes*. Eds. Litwack, G. and D. Krieger. Wiley & Sons Inc. New York 1964, 1-37.
13. RICE, T. R. and M. W. P. Transport of amino acids into the oestrogen primed uterus. Enhancement of the uptake by postnuclear incubation. *Biochem. J.* 1972, 128, 19-27.
14. RICE, T. R., M. W. P. and J. L. W. F. Transport of amino acids into the oestrogen primed uterus. *Biochem. biophys. Acta (Amst.)* 1968, 192, 9-103.
15. RICE, T. R., M. W. P. and J. L. W. F. Transport of amino acids into the oestrogen primed uterus. II. Effect of the level of extracellular sodium ion. *J. Biol. Chem.* 1972, 247, 7128-7134.
16. WILKES, M. B., A. L. L. SINCE and R. L. HAZELWOOD, Amino acid transport by the rat uterus during the estrous cycle and pregnancy. *Biol. of Reprod.* 1975, 12, 383-388.
17. WILKES, W. A. A comparison of the efflux rates of AIB from kidney cortex slices of mature and newborn rats. *Canad. J. Physiol. Pharmacol.* 1969, 46, 765-768.
18. WILKES, W. A. and J. A. CANN, A comparison of the amino acid concentrating ability of the kidney cortex of newborn and mature rats. *Canad. J. Physiol. Pharmacol.* 1968, 46, 165-169.

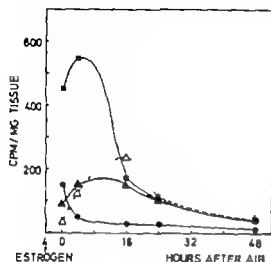


Fig. 3. Retention time of  $^{14}\text{C}$  AIB in blood (●—), diaphragm muscle (○—), brain (△---) and uterine tissue (■—).  $^{14}\text{C}$  AIB was administered 4 h after estrogen initiation of implantation.  $\text{SEM} = \text{max} \pm 50 \text{ cpm}$

### Discussion

Mice in delayed implantation which in addition to progesterone had received estrogen 4 or 8 h before being given  $^{14}\text{C}$  AIB *lv* displayed a high uterine uptake compared with mice not having received estrogen. Furthermore this uptake was preferential for the uterine tissue compared with some other tissues. Since the  $^{14}\text{C}$  AIB transport was considerably lower when only  $^{14}\text{C}$  AIB was given pretreatment *in vitro* with estrogen is necessary for the enhanced  $^{14}\text{C}$  AIB-accumulation. This is in agreement with the findings of Riggs *et al* (1968) for the *in vitro* uptake of AIB in immature rat uterus after *in vivo* preincubation with estradiol. Since the estrogen effect is not momentaneous it seems probable that the hormone might induce a *de novo* synthesis of AIB-carriers in the plasma membranes of uterine tissue. Such a proposal can be further supported by the determination of the  $V_{\text{max}}$  parameter and Riggs *et al* (1968) have shown that in their experimental system  $V_{\text{max}}$  increased while  $K_m$  remained unchanged by the treatment with estradiol.

AIB has been shown to be transported mainly by the route defined as the system A by Oxender and Christensen (1963). Several other amino acids as alanine, methionine and nor leucine are mainly transported by this route in different cells. Characteristical features for this system are the Na<sup>+</sup>-dependency and transport against a concentration gradient. This means necessarily that the exit rate by this system is slower than the entrance rate (Christensen and Handlogten 1968) and that the carrier operates asymmetrically. By this system it is therefore possible to augment the free intracellular amino acid pool while an exchange system can only influence the qualitative composition of the intracellular amino acid pool without enhancing the net uptake. Such an asymmetry has been shown to exist in slices of newborn rat kidney cortex while absent in adult cortex (Webber 1968, Webber and Cairns 1968).

The strict Na<sup>+</sup>-dependence of the AIB-uptake into uterine tissue can naturally not be demonstrated by *in vivo* expts. However Riggs *et al* (1972) have shown that the uptake of AIB into the estrogen primed rat uterus *in vitro* increased regularly with increasing Na<sup>+</sup> amounts in the incubation buffer. Thus we have reasons to believe that the system A is operating also *in vivo* in mice.

During activation for implantation the first 8 h are a period when system A amino acids are supplied from the blood into the uterine tissue at a high degree. Perhaps this accumulation of amino acids in the uterine tissue can be exploited also by the developing blastocyst. so, AIB should accumulate also in the blastocysts. This possibility is presently being tested.

This investigation was supported by grants from the Swedish Medical Research Council, project no B76-1X-22-12A and no 12X-70. Technical assistant: Mrs Barbro Elmqvist.

## References

1. CRICKER, H. N., *Biological Transport*, 2nd ed. 1973. W. A. Benjamin, Inc. Reading, Mass., U.S.A.
2. CRICKER, H. N. and M. E. HANLON, Modes of Mediated Excretion of Amino Acids from the Ehrlich Ascites Tumor Cells. *J. Biol. Chem.* 1968. 243: 3428-3438.
3. JAFFE, J. B. and S. J. PATE, The uptake of amino acids by mouse cells (strain L8) during growth in batch culture and chemically defined media: the influence of cell growth rate. *Proc. roy. Soc. B.* 1967. 168, 421-438.
4. LIVERSIDE, K. W. The induction of implantation in the mouse after ovariectomy. *Steroids* 1967. 10, 581-601.
5. MCCOY, D. J. and G. E. MULLER, Effect of estradiol on the level of amino acid-activating enzymes in the rat uterus. *J. Biol. Chem.* 1958. 232. 31-42.
6. NOLLE, M. W. and W. M. ALLAN, Early stimulation by estradiol of amino acid penetration in rabbit uterus. *J. Biol. Chem.* 1961. 236. 2967-2992.
7. NOLLE, M. W. T. R. RIGGS, L. M. WALLER and H. N. CHRISTENSEN, Endocrine control of amino acid transfer. *Science* 1957. 126. 1002-1003.
8. OLSEN, W. H., Brain uptake of radiolabeled amino acids, amines and hexoses after arterial injection. *Amer. J. Physiol.* 1971. 221. 1629-1639.
9. OLSEN, W. H. Stereospecificity of blood-brain barrier permeability to amino acids. *Amer. J. Physiol.* 1972. 224. 967-969.
10. OLSEN, D. L. and H. N. CHRISTENSEN, Distinct membrane systems for the transport of neutral amino acids by the Ehrlich cell. *J. Biol. Chem.* 1963. 238. 3466-3469.
11. PETERLIN, P. P. ROSSI, and E.-E. BALDINI, Steroid-induced early protein synthesis in rat uterus and prostate. *Europ. J. Biochem.* 1972. 30. 137-143.
12. RACE, T. R. *Action of hormones on molecular processes*. Ed. Lomax, G. and D. Kritzberg. Wiley & Sons Inc., New York 1964. 1-57.
13. RACE, T. R. and M. W. P., Transport of amino acids into the estrogen primed uterus. Enhancement of the uptake by preliminary incubation. *Biochem. J.* 1972. 126. 19-22.
14. RACE, T. R. M. W. P. and H. W. FEVO, Transport of amino acids into the estrogen primed uterus. *Biochem. biophys. Acta (Amst.)* 1968. 150. 81-103.
15. RACE, T. R. M. W. P. and H. W. FEVO, Transport of amino acids into the estrogen primed uterus. II. Effect of the level of extracellular sodium ion. *J. Biol. Chem.* 1972. 247. 7128-7134.
16. WATSON, M. R. A. L. LA. SINCE and R. L. HAZELWOOD, Amino acid transport by the rat uterus during the oestrous cycle and pregnancy. *Intl. J. Reprod.* 1975. 12. 383-388.
17. WILSON, B. A., A comparison of the efflux rates of AIB from kidney cortex of infant and newborn rats. *Canad. J. Physiol. Pharmacol.* 1968. 46. 765-768.
18. WILSON, B. A. and J. A. CAMPA, A comparison of the amino acid content of newborn and mature rats. *Canad. J. Physiol. Pharmacol.* 1968. 46. 1160-1168.

## Intra- and Extracellular Activity of Ciliated Cells

By

D. HUBERMAN, C. H. HÅKANSSON, U. MERCKE and N. G. TOREMÄLM

Received 13 May 1976

---

### Abstract

---

HUBERMAN, D., C. H. HÅKANSSON, U. MERCKE and N. G. TOREMÄLM. *Intra- and extracellular activity of ciliated cells*. Acta physiol. scand. 1977 99 42-47.

A method permitting simultaneous recording *in vitro* of the intracellular and extracellular (mucociliary) activity of respiratory ciliated mucous membrane is presented. Using this method the influence of different temperature levels (20°-30°-40°C) upon the tracheal mucous membrane of 15 rabbits has been investigated. It is shown 1) that the extracellular (= mucociliary) activity increases with rising temperature whereas the intracellular activity is unaffected and remains constant, indicating that temperature influences upon the respiratory tract mucus rather than a change of the intracellular "pace-maker" mechanism. 2) that the restraining effect of the mucus decreases with rising temperatures but is still 10% of the intracellular frequency at body temperature. 3) that the presented method should be useful for future investigations concerning the effect of air pollution and pharmacological substances upon respiratory mucous membranes.

**Key words:** Trachea, mucous membranes, ciliated cells, mucociliary activity, electrophysiological activity.

---

The mechanical defence mechanism of the respiratory mucous membrane is constituted of two factors—the propulsive movements of the cilia and the more or less restricting effect of the covering layer of mucus. The term mucociliary activity has therefore been found not adequate than the previously used designation, "the ciliary beat frequency" (Mercke *et al.* 1974 a). It has hitherto not been possible to separate the rhythm and frequency of intrinsic ciliary activity from that of the extrinsic mucociliary activity observed through a microscope, but such a functional difference should be of great practical importance for experimental and diagnostic purposes. However, this is not possible with the use of extracellular recording methods only. A combination of intra- and extracellular measurements is necessary.

The intracellular electrical activity in beating ciliated cells was first recorded in vertebrate by Håkansson and Toremalm in 1966. In recent experimental studies we found that the frequency of intracellular action potentials was about the same at 20°, 30° and 40°C during *in vitro* conditions while the extracellular movements of the cilia decreased considerably with decreasing temperatures (Toremalm *et al.* 1975). This means that the frequency change is

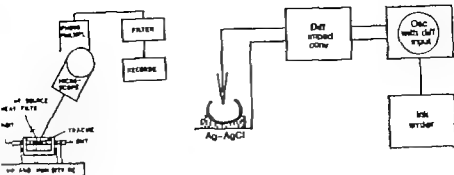


Fig. 1. Block diagram of the experimental equipment for recording mucociliary wave frequency (left) and intracellular activity (right).

lower temperatures is due to physical properties of the secretion layer and not to the intracellular "pace-maker" mechanism. However this conclusion was drawn from recordings of the intracellular and extracellular activities in separate specimens. Simultaneous recordings from the same area would be even more valuable for theoretical and practical purposes. The aim of the present investigation has therefore been: 1) to develop a method for the simultaneous recording of the intracellular electrical activity and the extracellular mucociliary wave frequency in the same area on tracheal specimens and 2) to compare the frequencies of the electrical discharge and the mucociliary movements at different temperature levels within the range of 20–40°C.

### Methods and material

Rabbits weighing 1.5–3 kg have been used as experimental animals. To avoid undesired pharmacological side effects they have been sacrificed by a blow on their heads. After careful dissection of the trachea a 5–10 cm specimen has been removed and put into an experimental chamber in which temperature and relative humidity can be regulated and recorded.

#### Recording of the mucociliary activity

The recording technique has already been extensively described (Morris *et al.* 1974 a). Briefly: cold light beam directed against the tracheal ciliated mucous membrane through slit in the upwards directed perspex membrane. Variations of the light intensity of the mucosal reflex are picked up by microscope (Fig. 1). A photoreceptor (EMI 9324 B) attached to one of the oculars converts the light intensity variations to electrical signals. Both after amplification and filtering (Krohn-Hite type 3550) are recorded by an ink writer (Lectra-Micrograph 34).

#### Recording of the intracellular electrical activity

The technique of recording is a modification of the method originally described by Håkansson and Torstam in 1966 and has in detail been described by Torstam *et al.* (1973). In the actual investigation further improvement has been introduced by exchanging the formerly used transistorized impedance converter to a low frequency impedance converter with resistance of 1000 MΩ and an amplification of 1:1 (Fig. 1).

#### Experimental procedure

After the tracheal specimen has been placed into the experimental chamber the capillary microelectrode is on slowly pushed downwards through the mucus into the cell layer in the center of the light reflex. The mucus potential is the DC potential. When potential change of 15 to 40 mV indicates that ciliated

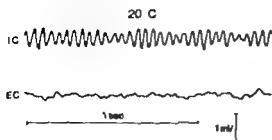


Fig. 2. Recordings from one tracheal specimen showing the intracellular activity (IC) and extracellular mucociliary wave patterns (EC) at 20°C.

cell membrane has been penetrated, the oscilloscope is changed to the AC position and the above described intracellular recording is commenced. The microscope is then adjusted so that variations of the light reflections exactly corresponding to the area of intracellular recording may be picked up and recorded.

The simultaneous recording of intracellular electrical and extracellular mechanical activities from a very small mucosal area has been performed at 3 different temperature levels (20°, 30° and 40°C) and at an environmental air humidity above 90%. The trachea from 5 rabbits have been investigated at each temperature level.

### Results

At 20°C (Fig. 2) the intracellular potential recordings show a fairly even rhythm and frequency of 20 cycles/s whereas the extracellular recordings are irregular with a low frequency of about 12 waves/s (Table I).

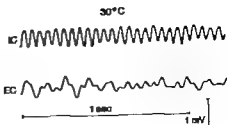
At 30°C (Fig. 3) the intracellular frequency does not change in contrast to the extracellular activity which has increased. The difference in the intra- and extracellular activities

TABLE I

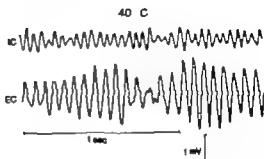
Temp. (°C)	Intracellular activity (oscillations/s)	Extracellular (mucociliary) activity (waves/s)
20	20.5	9.6
	21.5	13.1
	21.0	17.1
	21.7	10.4
	21.3	12.7
	M 21.2	M 12.6
30	21.3	16.7
	21.5	17.4
	23.1	18.0
	21.4	17.6
	20.8	16.3
	M 21.6	M 17.2
40	22.1	18.8
	22.0	19.5
	21.5	17.7
	21.2	18.8
	21.5	17.4



3. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary pattern (EC) at 30°C.



4. Recordings from one tracheal specimen showing the intracellular activity (IC) and the mucociliary pattern (EC) at 40°C.



slightly reduced (20 cycles and 17 waves/s respectively Table I). At 40°C (Fig. 4) this difference is even more reduced, now being 20 cycles and 18 waves/s respectively (Table I). The frequency/temperature relationship concerning the intracellular as well as the extracellular recordings is illustrated in Fig. 5. The plotted values are the mean frequencies of 15 rabbit specimens at each temperature level (Table I). From this figure it is clearly evident that the intracellular activity is mainly unaffected by temperature changes in the interval 20°C to 40°C. However the extracellular or mucociliary activity increases almost linearly with increasing temperature in the same interval.

### Discussion

A comparative study of the intracellular electrical activity which initiates the movements of the cilia covering the surface of the respiratory tract, and the extracellular manifestations

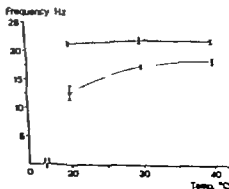


Fig. 5. The frequency-temperature relationship regarding intracellular (broken line) and extracellular (solid line) activities simultaneously measured. Each plotted value is the average of 5 measurements.

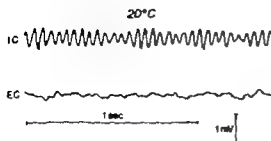


Fig. 2. Recordings from one tracheal spot showing the intracellular activity (IC) and mucociliary wave pattern (EC) at 20°C.

cell membrane has been penetrated, the oscilloscope is changed to the AC position and the intracellular recording is commenced. The microscope is then adjusted so that variations of the light intensities exactly corresponding to the area of intracellular recording may be picked up and recorded.

The simultaneous recording of intracellular electrical and extracellular mechanical activities from very small mucosal area has been performed at 3 different temperature levels (20°, 30° and 40°C) in an environmental air humidity above 90%. The trachea from 5 rabbits have been investigated at temperature level.

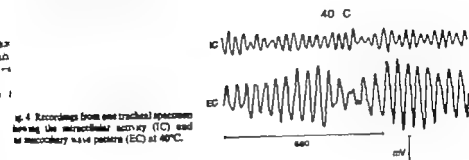
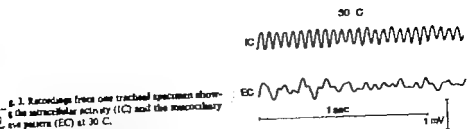
## Results

At 20°C (Fig. 2) the intracellular potential recordings show a fairly even rhythm at frequency of 20 cycles/s whereas the extracellular recordings are irregular with a low frequency of about 12 waves/s (Table 1).

At 30°C (Fig. 3) the intracellular frequency does not change in contrast to the extracellular activity which has increased. The difference in the intra- and extracellular activities

Table 1

Temp. (°C)	Intracellular activity (oscillations/s)	Extracellular (mucociliary) activity (waves/s)
20	20.3	9.6
	21.5	13.1
	21.0	17.1
	21.7	10.4
	21.3	12.7
	M 21.2	M 12.6
30	21.3	16.7
	21.5	17.4
	21.1	18.0
	21.4	17.6
	20.8	16.3
	M 21.6	M 17.2
40	22.1	18.8
	22.0	19.5
	21.5	17.7
	21.2	18.8
	21.5	17.4
	M 21.5	M 18.4



markedly reduced (20 cycles and 17 waves/s respectively Table I). At 40 C (Fig. 4) this difference is even more reduced, now being 20 cycles and 18 waves/s respectively (Table I).

The frequency-temperature relationship concerning the intracellular as well as the extracellular recordings is illustrated in Fig. 5. The plotted values are the mean frequencies of all 5 rabbit specimens at each temperature level (Table I). From this figure it is clearly evident that the intracellular activity is mainly unaffected by temperature changes in the interval 20 C to 40 C. However the extracellular or macrociliary activity increases almost linearly with increasing temperature in the same interval.

### Discussion

A comparative study of the intracellular electrical activity which initiates the movements of the cilia covering the surface of the respiratory tract, and the extracellular manifestations

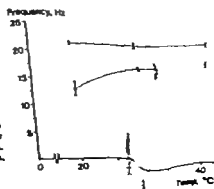


Fig. 5. The frequency-temperature relationship for the extracellular (broken line) and intracellular (solid line) activities simultaneously measured. Each point value is the average of 5 measurements.

of this activity is presented in this investigation. The experimental model is a comb of two previously described methods (Fig. 1) dealing with one of these activities at (Mercke *et al.* 1974 a, Toremalm *et al.* 1975). It has been possible to make simultaneous recordings of the intracellular electrical activity in one ciliated cell and the extracellular mechanical activity from a small area covering about 10 ciliated cells during standard conditions regarding temperature and humidity of the surrounding air.

This investigation has been limited to a comparison of the two mentioned activities at three different temperature levels. Typical recordings at 20°, 30° and 40° C are demonstrated in Fig. 2, 3 and 4 respectively. The entire results regarding all tracheal specimens are in Table 1 and are also graphically illustrated in Fig. 5.

The relative constancy of the intracellular oscillations from 20 to 40°C is a physiologically interesting phenomenon. In fact this may have two explanations: 1. The rhythm "pace-maker" is relatively insensitive to temperature changes. 2. The frequency is constant but the rising time of the potential fluctuation can be shorter and the rise rapid at higher temperatures. This may result in an increasing amplitude of the intracellular recording. Such an explanation cannot be deduced from Fig. 2, 3 and 4 since the recordings at 20, 30 and 40° C are obtained from different tracheal specimens. To be able to test the hypothesis further, experiments have to be done where one tracheal specimen is investigated at the different temperatures.

The extracellular mucociliary activity on the other hand is strongly influenced by temperature changes. This has been described more in detail in two previous papers (Mercke 1974 b, Mercke 1974). From the present simultaneous recordings frequency differences between the intra- and extracellular activities increase by decreasing temperature below 40° C (Fig. 5). Ordinary body temperature therefore seems to be optimal for the cilia to work in. In other words the mechanical restriction of the secretion layer is minimal at this temperature level. However, even at optimal temperature conditions there is a difference of about 2 cycles/s between the internal and external activities. It means that the secretion layer normally has a decelerating effect upon the movements of cilia. The consequences of the present results are (1) that the method makes it possible to separate the intracellular ciliary activity (the "pace-maker" effect) from the retarding effect of the mucus layer surrounding the cilia, and (2) that this inhibitory factor reduces the frequency by about 10% above body temperature.

This knowledge seems to be valuable for future experimental investigations in which the model may be used e.g. for air pollution tests and control of pharmacological substances for local treatment of the respiratory tract. From the results a hypothetical conclusion can be drawn implying that it must be easier to stimulate the mucociliary defence mechanism by changing the physical properties of the mucus than to try to accelerate the intracellular activity of the ciliated cells. However, this hypothesis needs to be proven by a series of further experiments which are now in progress.

## References

- ILANSON, C. H. and N. G. TORMMALM, Studies on the physiology of the trachea. III. Electrical activity of the ciliary cell layer. *Ann. Otol.* (St. Louis) 1966, 75: 1007-1018.
- LECK, U. The influence of temperature on mucociliary activity. Temperature range 40°C-50°C. *Acta oto-laryng.* (Stockh.) 1974, 78: 253-258.
- LECK, U., C. H. ILANSON and N. G. TORMMALM, A method for standardized studies of mucociliary activity. *Acta oto-laryng.* (Stockh.) 1974 a, 78: 118-123.
- LECK, U., C. H. ILANSON and N. G. TORMMALM, The influence of temperature on mucociliary activity. Temperature range 20°C-40°C. *Acta oto-laryng.* (Stockh.) 1974 b, 78: 444-450.
- TORMMALM, N. G., C. H. ILANSON, U. LECK, B. DAMERLUS and D. HUMERMAN, Mucociliary activity. A comparative analysis of intracellular and extracellular activity. *Acta oto-laryng.* (Stockh.) 1975, 79: 434-441.

## The Effect of Teeth Amputations on the Choline Acetyltransferase Activity of Rat Submaxillary Glands

By

JÖRGEN EKSTRÖM

Received 21 May 1976

---

### Abstract

Ekström, J. *The effect of teeth amputations on the choline acetyltransferase activity of rat submaxillary glands* Acta physiol. scand. 1977 99 48-52.

The choline acetyltransferase activity in parasympathetically decentralized glands was unaffected by repeated teeth amputations over a period of 2 weeks, while after the same period of time the treatment caused the enzyme activity to increase in innervated glands. It appears that the enzyme in the postganglionic nerve is for its activity dependent on an intact connection with the central nervous system. The increase in enzyme activity is attributed to an enhanced reflex stimulation of the glands from pulpal receptors.

*Key words:* Choline acetyltransferase activity, salivary glands, impulse flow

---

When the parasympathetic postganglionic neurones of the salivary glands lose their connection with the central nervous system by a section of the preganglionic nerve supply their capacity to synthesize acetylcholine has been found to diminish, as judged from the fall in the activity of choline acetyltransferase (Nordenfelt 1964, Ekström and Holmberg 1972). Based on this observation Nordenfelt (1964, 1965) suggested that the activity of the acetylcholine forming enzyme might be influenced by the intensity of the impulse traffic in the neurones, as to the adrenergic neurones a similar relationship between the activity of catecholamine synthesizing enzymes and impulse traffic has since been considered (Zieher and Pellegrino de Iraldi 1966, Molinoff and Axelrod 1971, Thoenen 1974). The idea put forward by Nordenfelt is favoured by the outcome of various types of experiments on salivary glands, where the degree to which the glands were activated from the central nervous system was either decreased or increased over a long time period (see Ekström 1975). In parotid glands of rats the choline acetyltransferase activity declined when the animals were kept on a liquid diet, while it increased when the diet was dry and bulky or when the mouth and the throat of the animals were made dry by atropinization or salivary duct ligations. The increase in the choline acetyltransferase activity in the submaxillary glands of the rat after repeated teeth amputations reported by Ohlin and Perac (1967) seems also to be a finding, which would fit into the pattern outlined above: the enzyme activity is increased as a consequent

the enhanced stimulation of the glands reflexly elicited by the irritation of pulpal receptors. However, these authors reported that in the parasympathetically decentralized glands the acetylcholinesterase activity had increased after the amputations. This might suggest some other factor than the impulse traffic is responsible for the increased enzyme activity after teeth amputations.

In the present study the effect of teeth amputations on the choline acetyltransferase activity was compared in decentralized and innervated submaxillary glands.

## Methods

Male rats of Sprague-Dawley strain bred at this institute were used. They were at the start of the experiments 4 months old. The diet used was of granulated consistency prepared daily by mixing powder made of standard pelleted diet with water, so as to make it easier for the rats with amputated teeth to eat; the rats serving as controls were also given this diet. The diet and water were given *ad libitum*. Both at the start and at the end of the experimental period the body weights of the rats were determined.

A rat lightly anesthetized with ether both the lower and upper incisors were cut to the gingival margin with bone cutting forceps every other day over a period of either 14 or 21 days. The rats serving as controls were also exposed to ether for a few minutes at the same occasions.

In one type of experiment the chorda-buccal nerve was bilaterally cut under dissecting microscope in 18 rats, and a few mm of the nerve was removed. The wound was then sutured. 19 of these rats (teeth amputations started 7 days later and continued over a time period of 14 days, the other 9 rats were later mates to 7 amputated ones and served as controls. In another type of experiment with an intact innervation of these lower glands were exposed to the amputations. In the first series, the incisors of 9 rats were amputated over a period of 14 days, in a second series 7 rats were amputated over a period of 21 days, 9 and 7 unoperated litter mates to those rats were amputated served as controls.

At the end of the experimental period the rats were killed by inhalation of ether and the submaxillary glands were removed (the sublingual glands being separated from the submaxillaries), washed in saline, pinned, pressed gently between glass plate and weighed, so the rats studied over the period of 3 weeks in sublingual glands were also removed and treated in the same way as the submaxillaries. Both wet and dry weights were determined, the latter expressed as acetone dried powder.

The glands were then prepared for the analysis of their activity of choline acetyltransferase using the method devised by Hesse (see Nordenskjöld 1963, 1965). Acetone dried powder as made of the 2 pooled submaxillary glands of one rat, so the sublingual glands each pool consisted of the glands of 7 rats. The powder of the glands was made up in cytochrome-calcium in a concentration of 50 mg/ml. Of the tissue extract 0.2 ml was incubated at 38°C for 60 min. Experimental preparation and control preparation were incubated simultaneously and the assay for acetylcholine was made on the same acetylated frog rectus. The choline acetyltransferase activity is expressed as  $\mu\text{g}$  acetylcholine chloride formed per h per pooled glands (total activity) and as  $\mu\text{g}$  acetylcholine chloride formed per h per g acetone per dry (concentration).

Student's *t*-test as used, paired comparisons were made between the experimental rat and its control litter mate. *P* values of less than 0.05 were considered significant.

## Results

**Body weights.** At the start of the experiments no differences existed in body weights between the rats to be used as controls and those to be teeth amputated. The body weights of the teeth amputated rats with decentralized glands decreased ( $p < 0.001$ ) during the 14 days from mean (S.E.) 294.8 to 259.8 g (n = 9), i.e. by 12%. During the same time period the control litter mates increased ( $p < 0.001$ ) in weights by 7%, from 293.9 to 314.9 g (n = 9). The body weights of the teeth amputated rats with an intact innervation decreased from 301.7 to 267.8 g (n = 9), i.e. by 11% in the experiment of the duration of 14 days and in the experiment